Bioleaching im Lausitzer und Mitteldeutschen Braunkohlerevier:

Mikrobiologische Bestandsaufnahme und Laborexperimente zur Hemmung der mikrobiellen Pyrit-Oxidation

Dissertation

zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

– Dr. rer. nat. –

vorgelegt von

Hans-Michael Siebert

geboren in Hoyerswerda

Universität Duisburg-Essen

2012

Die vorliegende kumulative Dissertation wurde im Zeitraum von Februar 2008 bis August 2012 in der Arbeitsgruppe Technische Mikrobiologie von Prof. Dr. Stahmann an der Naturwissenschaftlichen Fakultät der Hochschule Lausitz (FH) in Zusammenarbeit mit dem Arbeitskreis Aquatische Biotechnologie von Prof. Dr. Sand an der Fakultät für Chemie der Universität Duisburg- Essen durchgeführt.

Tag der Disputation: 30. August 2012

Gutachter:	Prof. Dr. Wolfgang Sand
	Prof. Dr. Klaus-Peter Stahmann
Vorsitzender:	Prof. Dr. Torsten Schmidt

Zusammenfassung

In der Lausitz und in Mitteldeutschland wird seit Anfang des 20. Jahrhunderts Braunkohle abgebaut. Durch diesen Abbau entstanden aus den geschlossenen Tagebauen Restseen, die durch einen sauren pH- Wert und eine hohe Sulfatlast charakterisiert sind. Diese Versauerung wird „Acid Mine Drainage“ (AMD) genannt. Ziel der Arbeit war der Nachweis von azidophilen Laugungsmikroorganismen im Lausitzer und Mitteldeutschen Braunkohlerevier. Es sollte ein Zusammenhang zwischen diesen Mikroorganismen und dem AMD gezeigt, eine Hemmung des AMD im Labor erzielt und der Wirkmechanismus auf die Mikroorganismen erklärt werden.

Aus 66 Bodenproben, die 2006 und 2008 aus drei Lausitzer und zwei mitteldeutschen Braunkohletagebauen gewonnen wurden, konnten Eisen- und Schwefel- oxidierende Mikroorganismen durch Kultivierung bei 28 bis 70°C isoliert werden. Aus 168 Reinkulturen wurden 12 verschiedene Spezies, wie z.B. *Acidithiobacillus ferrooxidans*, *A. thiooxidans*, *Leptospirillum ferrooxidans*, *L. ferriphilum*, *Ferrimicrobium acidiphilum*, *Acidiphilium acidophilum* und *Sulfobacillus thermosulfidooxidans*, nachgewiesen. Nach Extraktion der Nukleinsäuren direkt aus den Proben und Aufreinigung von PCR- inhibierenden Substanzen wurden diese Ergebnisse durch spezifische Amplifikate der 16S rRNA Gene für *A. ferrooxidans*, *A. thiooxidans* und *Leptospirillum* spp. unterstützt. Weiterhin konnte der Nachweis der mikrobiellen Stoffwechselaktivität durch Mikrokalorimetrie erbracht werden. Durch Untersuchung von 42 Bohrkernproben nach und 24 Bohrkernproben vor der Absenkung des Grundwassers, genommen 2008 und 2009, konnte die Theorie aufgestellt werden, dass die biologische Pyritoxidation mit Absenkung des Grundwassers, d.h. dem Einströmen von Luftsauerstoff, beginnt.

Für die Hemmung der Eisen- und Schwefel- oxidierenden Mikroorganismen wurden Säulenexperimente im 20 mL und 20 L Maßstab, sowie in Temperaturbereichen von 12 bis 25°C und mit verschiedenen Tensid- Regimen durchgeführt. Das Bioleaching konnte durch Zugabe von Regenwasser gestartet werden. Durch SDS- oder Texapon N70- Zugaben konnte in 20 mL Säulen für bis zu 38 Wochen eine pH Anhebung und Abtötung der Eisen- und Schwefel-oxidierenden Mikroorganismen gemessen werden. Dabei wurde eine Verringerung der Sulfat- Freisetzung um bis minus 70% gemessen. In Säulen im 20 L Maßstab, betrieben bei konstant 12°C oder 15 bis 21°C, wurde die biologische Laugung gestartet und anschließend für bis zu 45 Wochen gehemmt, messbar durch eine pH- Wert Anhebung, das Vorkommen von vorzugsweise Eisen (II) im Durchlauf der Säulen und die Abtötung der Eisen- und Schwefel-oxidierenden Mikroorganismen. Die Sulfat- Auswaschung wurde um 29 bis 44% verringert.

In Untersuchungen mit konzentrierten Suspensionen von *A. ferrooxidans*, *A. thiooxidans* und *Ac. cryptum* im Vergleich zu *Escherichia coli* und *Bacillus subtilis* konnte eine induzierte Lyse der Laugungsbakterien durch Behandlung mit SDS und Texapon N70 nachgewiesen werden. Für *A. thiooxidans* wurde eine Verringerung der Zellzahlen um bis zu 75% durch SDS Behandlung gezählt. Für die drei Laugungsbakterien wurde durch Behandlung mit den Tensiden SDS und Texapon N70 eine Verringerung der OD_{600} um bis zu 80% gemessen, wogegen für *E. coli* und *B. subtilis* nur eine Verringerung um 10 bis 20% gefunden wurde. Für alle Bakterien wurde eine Freisetzung von DNA und RNA gefunden, eine Freisetzung von 50 bis 80% der Gesamtproteine wurde für die Laugungsbakterien gemessen, für *E. coli* dagegen nur 0,35%. Diese Ergebnisse sprechen für eine induzierte Lyse als Wirkmechanismus und erklären den Erfolg der Hemmung des Bioleaching in den Säulenversuchen.

Abstract

Since the beginning of the 20th century in Lusatia and in Central Germany the mining of lignite was performed. Therefore mining lakes exist and are characterised by a low pH value and increased sulphate concentrations. This process is harmful to the environment and called acid mine drainage (AMD). Aim of this study was the detection of acidophilic microorganisms in the mining areas in Lusatia and in central Germany. Furthermore, the connection between these leaching microorganisms and the AMD, the inhibition of AMD in the laboratory scale, and the mechanism of action of the surfactants to the microorganisms were examined.

From 66 sand samples, taken in 2006 and 2008, from three lignite mining areas in Lusatia and from two mining areas in central Germany iron- and sulphur-oxidizing microorganisms were detected by cultivation based technique at temperatures from 28 to 70°C. From 168 isolated pure cultures, the microorganisms were identified by sequencing of the 16S rRNA genes as 12 different species e.g. *Acidithiobacillus ferrooxidans, A. thiooxidans, Leptospirillum ferrooxidans, L. ferriphilum, Ferrimicrobium acidiphilum, Acidiphilium acidophilum* and *Sulfobacillus thermosulfidooxidans*. By the amplification of the 16S rRNA genes after extraction of the nucleic acids direct from the sand and purification from PCR inhibiting substances the species *A. ferrooxidans, A. thiooxidans* and *Leptospirillum* spp. were detected and supported the results derived from the classical method. Furthermore, microcalorimetric determination of the metabolic activity supported the results shown above. After examination of 42 drilling core samples after lowering the groundwater and 24 samples of drilling cores before lowering the ground water, taken in 2008 and 2009, the hypothesis was proposed that the biological pyrite oxidation starts by lowering the ground water and influx of air.

For the development of a strategy for the inhibition of leaching microorganisms, experiments were performed in columns in 20 mL and 20 L scale, filled with aquifer material, in the temperature range from 12 to 25°C and different surfactant regimes. The leaching was started by the addition of rainwater to the columns. By addition of SDS or Texapon N 70 an inhibition of the bioleaching was achieved in the 20 mL columns for up to 38 weeks. This was measured by an increased pH, missing detection of iron- and sulphur- oxidizing microorganisms in sand or solution. At all temperatures a reduction of the sulphate concentration was found, with a maximum of minus 70%. In 20 L columns, operated at constant 12°C or from 15 to 21°C, the bioleaching was successfully initiated and afterwards inhibited by surfactant treatment for up to 45 weeks. The surfactant addition achieved an inhibition of iron- and sulphur-oxidizing microorganisms, hence the occurrence of iron in the reduced form and an increasing pH of the leaching solution. Furthermore, the total sulphate release was decreased between 29 and 44% in comparison to the rainwater washed control, respectively.

By examinations of concentrated suspensions of *A. ferrooxidans*, *A. thiooxidans* and *Ac. cryptum* in comparison to *E. coli* and *B. subtilis* an induced lysis of the leaching bacteria by SDS and Texapon N70 treatment was observed. By SDS treatment a decrease in the cell count down to minus 75% for *A. thiooxidans* was found. Surfactant treatment of the three leaching bacteria caused a decrease in the optical density at 600nm by 80%, whereas only a slight decrease between 10 and 20% was measured for *E. coli* and *B. subtilis*, respectively. For all bacteria a release of DNA and RNA was found. Furthermore, a release of 50 to 80% of total protein content was measured for the leaching bacteria after the SDS treatment, whereas for *E. coli* only 0.35% was detected. These results indicate an induced lyis mechanism and explain the successful inhibition of the bioleaching in the columns by SDS and Texapon N70 addition.

Inhaltsverzeichnis

1. Einleitung

1.1 Acid Mine Drainage

Braun- und Steinkohle werden weltweit abgebaut. Im Jahr 2010 waren die größten Produzenten China, USA, Australien, Indien und Indonesien (Rutledge, 2011). In Deutschland wird Braunkohle in den Revieren im Rheinland, in Mitteldeutschland südlich von Leipzig und im Lausitzer Revier in Sachsen und Brandenburg abgebaut (Schultze *et al.*, 2010). Die Anfänge im Lausitzer Revier können bis ins 17. Jahrhundert zurückverfolgt werden. Der Abbau im großtechnischen Maßstab wird seit Anfang des 20. Jahrhunderts betrieben (Heitmann *et al.*, 2010). Resultierend aus dem langjährigen Bergbau sind Tagebaurestseen entstanden, welche durch einen pH- Wert unter 4 und einen hohen Ionen- Gehalt mit Sulfat als Haupt-Ion charakterisiert sind (Herzsprung *et al.*, 1998; Geller *et al.*, 1998).

Die Versauerung des Grund-, Sicker- und Oberflächenwassers wird „Acid Mine Drainage“ (AMD) genannt. Sie entsteht durch Oxidation von schwefelhaltigen Mineralien (Metallsulfiden), wie Pyrit, Markasit oder Chalkopyrit (Evangelou, 1995). Die schwefelsauren Abbauprodukte können weitere Schwermetalle aus Mineralien lösen und so eine Verunreinigung des Grund- und Oberflächenwassers verursachen.
Für den Braunkohlebergbau wurde die Pyritoxidation oder AMD Bildung als zweistufiger und vor allem chemischer Prozess beschrieben. Die Oxidation von Pyrit (FeS_2) durch Luftsauerstoff kann mit Gleichung (1) beschrieben werden.

$$FeS_2 + 3½\ O_2 + H_2O \rightarrow Fe^{2+} + 2\ SO_4^{2-} + 2\ H^+ \quad (1)$$

Die Oxidation von Eisensulfiden zwischen der Abbaukante des Deckgebirges und der Oberfläche der Kippe wurde dabei als Primäroxidation bezeichnet

(Wisotzky, 1994; Wisotzky und Obermann, 2001). Der zweite Schritt, die Sekundäroxidation, findet in den rekultivierten Kippen statt. In den hier oben zitierten Untersuchungen wurde das Hauptaugenmerk auf die chemischen Prozesse gelegt. Der Einfluss von Eisen- und Schwefel- oxidierenden Mikroorganismen auf diese Prozesse ist aber seit langem bekannt (Colmer und Hinkle, 1947; Evangelou, 1995; Evangelou, 1998; Singer und Stumm, 1970)

1.2 Azidophile Mikroorganismen in Acid Mine Drainage Gebieten

Es ist bekannt das azidophile Mikroorganismen Metallsulfide unter aeroben Bedingungen angreifen können (Temple und Colmer, 1951; Kelly und Wood, 2000; Rohwerder *et al*. 2003), wobei zwei verschiedene Mechanismen genutzt werden (Schippers und Sand, 1999). Mikroorganismen wirken dabei als Katalysator, wobei unter anderem die Reaktionsgeschwindigkeiten der Oxidation von Eisen (II) zu Eisen (III) wie auch die Pyritoxidation durch Eisen (III) um den Faktor drei bis zehn erhöht werden können.

$$Fe^{2+} + \tfrac{1}{4}\, O_2 + H^+ \rightarrow Fe^{3+} + \tfrac{1}{2}\, H_2O \quad (2)$$

$$FeS_2 + 14\, Fe^{3+} + 8\, H_2O \rightarrow 15\, Fe^{2+} + 2\, SO_4^{2-} + 16\, H^+ \quad (3)$$

Eine Vergrößerung der Eisen (III) Ionen Konzentration durch Komplexierung mit Glucuronsäuren im Biofilm von *Acidithiobacillus ferrooxidans* (Abb. 1) soll einen oxidativen Angriff auf das Metallsulfid ermöglichen, wodurch die Reaktionsgeschwindigkeit um den Faktor 20 bis 100 erhöht werden könnte (Sand und Gehrke, 2006).

Untersuchungen in bergbaulich beeinflussten Landschaften zeigten eine Diversität von 17 verschiedenen prokaryotischen Spezies (Rohwerder *et al*., 2003). Zu den bekanntesten Mikroorganismen zählen die vor vielen Jahrzehnten zum ersten Mal beschriebenen Eisen- und Schwefel- oxidierenden Bakterien *A.*

ferrooxidans und *A. thiooxidans* (Temple und Colmer, 1951; Waksman und Joffe, 1922; Kelly und Wood, 2000). Außer diesen mesophilen γ-Proteobakterien, wurden in diesen Habitaten unter anderem auch Spezies der Gattung *Leptospirillum* spp. oder *Acidiphilium* spp. identifiziert (Markosyan, 1972; Hippe, 2000; Küsel *et al.*, 1999). Neben den mesophilen Eisen- und Schwefel- oxidierenden wurden auch moderat thermophile Bakterien

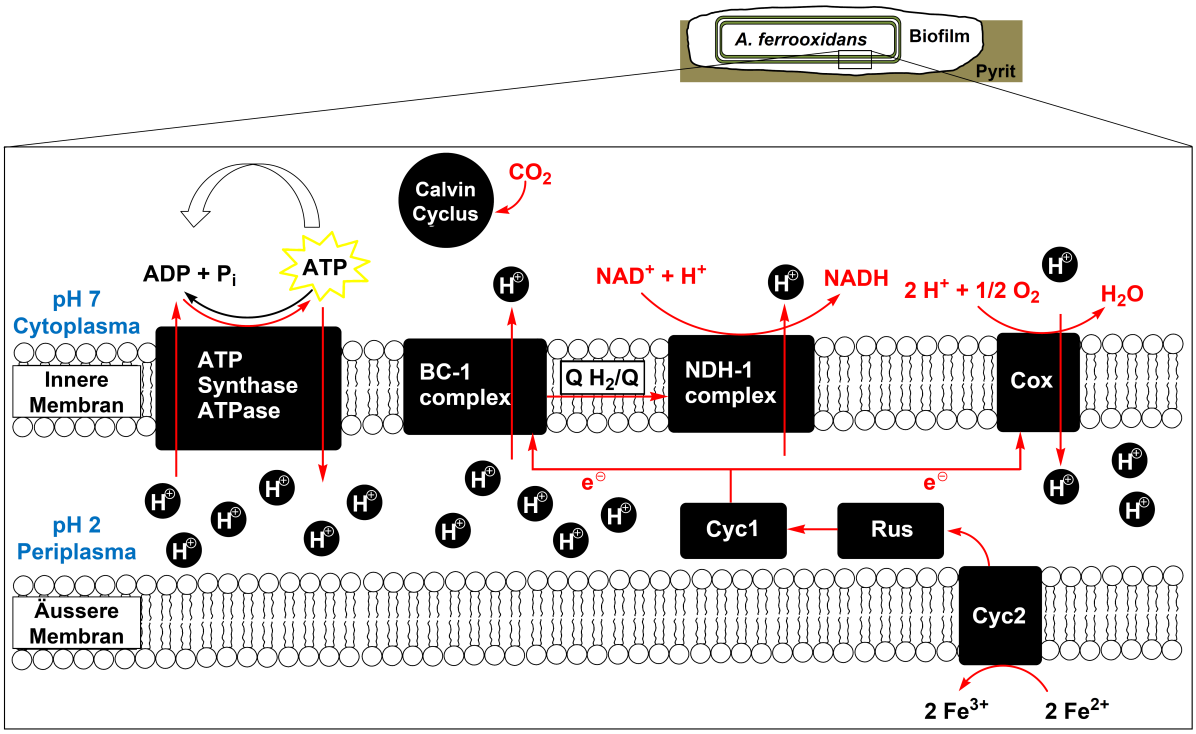

Abbildung 1. Zelle von *A. ferrooxidans*, die lokal Pyrit auflöst und Ausschnittsvergrößerung des relevanten Stoffwechsels. Dieses Gram-negative, azidophile, chemolithoautotrophe Bakterium gewinnt die benötigte Energie aus der Oxidation von Eisen (II) zu Eisen (III). Die Elektronen werden in zwei Stoffwechselwegen genutzt. Eine Möglichkeit ist die Elektronentransportkette, die einen Protonengradienten für die ATP Produktion erzeugt. Der zweite Weg ist ein endergonischer Elektronentransfer, der für die Reduktion von NAD^+ zu NADH+ H^+ benötigt wird. Dieses Coenzym wird im Calvin Zyklus für die Synthese von Hexosen benötigt (modifiziert nach Elbehti *et al.*, 2000; Rohwerder *et al.*, 2003)

gefunden, wie Spezies der Gattungen *Ferrimicrobium*, *Sulfobacillus* und *Acidimicrobium* (Johnson *et al.*, 2009; Norris *et al.*, 1996; Clark und Norris, 1996). Neben azidophilen Bakterien wurden auch Archaea detektiert. Diese wurden vor allem für extreme Habitate beschrieben, die sich z.B. durch Temperaturen von über 65°C auszeichnen. Zu diesen extrem thermophilen Eisen- und Schwefel- oxidierenden Archaea, z.B. aus der Ordnung *Sulfolobales*, gehören Spezies der Gattungen *Sulfolobus*, *Acidianus* und *Metallosphaera* (Fuchs *et al.*, 1995; Fuchs *et al.*, 1996; Norris *et al.*, 2000). Zusätzlich zu diesen

Beispielen für thermophile Archaea wurden auch moderat Thermophile, wie z.B. *Ferroplasma acidiphilum* oder *F. acidarmanus*, gefunden (Golyshina *et al*., 2000; Edwards *et al*., 2000).

1.3 Hemmung des Acid Mine Drainage

Um AMD zu hemmen, wurden verschiedene Rekultivierungs- oder Inhibitionstechniken beschrieben. Diese lassen sich in passive und aktive oder in chemische und biologische Techniken unterteilen (Johnson und Hallberg, 2005). Eine seit vielen Jahren bekannte passive Möglichkeit der AMD Behandlung ist die Nutzung von Kalkstein in „Anoxic Limestone Drains“ (ALD). Dabei sickert das Grubenwasser unter anaeroben Bedingungen, d.h. im Wasser gelöste Sauerstoff- Konzentration unter 1 mg/L, durch mit Kalkstein befüllte Gräben, wobei es zu einer pH Anhebung kommt (Hedin *et al*. 1994). Da bei dieser Behandlung die Konzentration von Schwermetallen und die hohe Sulfatlast im Grubenwasser nicht vermindert werden, sind weitere Methoden, z.B. „Constructed Wetlands“ nötig. Eine Abwandlung der ALD Methode ist die Nutzung von „Oxic Limestone Drains“ (OLD), wie von Cravotta und Trahan, 1999, beschrieben. Dabei wird Grubenwasser durch Vermischung mit Sauerstoff angereichert und über mit Kalkstein befüllte Röhren geleitet. Durch diese Methode werden pH und Alkalinität erhöht, wohingegen sich der Eisengehalt und der Gehalt an Aluminium- Ionen um 95% verringern. Eine weitere Alternative wäre z.B. die Behandlung des Grubenwassers mit Flugasche (Vadapalli *et al*., 2008). Dabei konnte der pH- Wert von 2,7 auf 11,5 angehoben und der Gehalt an Eisen- und Aluminium- Ionen reduziert werden. Durch Rekultivierung von bis zu 150 Jahre alten Kippen konnte eine Verringerung der Freisetzung von Sulfat oder Schwermetallen um 85 bis 99,98% erreicht werden (Willscher *et al*., 2010). Durch Sauerstoff verbrauchende, organische Substrate im Sickerwasser wurde dabei die Geschwindigkeit der Pyritoxidation verringert, da in diesem Milieu die Zellzahl Sulfat- reduzierender Mikroorganismen erhöht

ist. Die Nutzung von Sulfat- reduzierenden Mikroorganismen zur Verminderung des AMD wurde durch den Zusatz von Kohlenstoff- oder Energiequellen, wie H_2, Acetat, Ethanol oder komplexen organischen Substraten beschrieben (Koschorrek, 2008).

Da Eisen- und Schwefel- oxidierende Mikroorganismen die Geschwindigkeit der Pyritoxidation um den Faktor drei bis zehn beschleunigen, wurden Untersuchungen gemacht, den Katalysator, d.h. die Mikroorganismen, durch Biozide zu hemmen. Eine der Möglichkeiten ist die Nutzung von organischen Verbindungen, wie Detergentien, quartäre Ammonium- Verbindungen oder Isothiazolinon. Experimente mit planktonischen *A. ferrooxidans* Zellen mit dem Tensid Natriumlaurylsulfat (SDS) zeigten widersprüchliche Ergebnisse. Dabei wurden sowohl eine Erhöhung als auch eine Hemmung der biologischen Laugung beschrieben (Dugan und Apel, 1983; Onysoko *et al*., 1984; Sand, 1985; Dugan, 1986). Es wurde gezeigt, dass die Wirkung von SDS und anderen oberflächenaktiven Substanzen auf die Mikroorganismen auf der Lyse der Zytoplasmamembran oder auf der Hemmung der Adhäsion an die Substratoberfläche beruht (Jones, 1999; Neu 1996). Andere Substanzen, wie einfache organische, quartäre Ammonium- Verbindungen oder Kohlenwasserstoffe, zeigten eine erfolgreiche Hemmung planktonischer Mikroorganismen (Tuttle *et al*., 1976; Sikkema *et al*., 1995; Kourai *et al*., 2006; Sumitomo *et al*., 2006). Für Mikroorganismen, die im Biofilm leben, wurde dagegen nur eine eingeschränkte Wirksamkeit der Biozide beschrieben (Nett *et al*., 2008).

Der Einfluss von Bioziden auf Eisen- und Schwefel- oxidierende Mikroorganismen machte weitere Untersuchungen nötig, da diese in ihrem natürlichen Habitat, dem Boden, als planktonische Zellen und als Biofilm vorkommen. Weiterhin lassen auch andere Einflüsse, z.B. die Adhäsion des

Biozides an Bodenpartikel oder der Abbau durch Mikroorganismen auf eine verringerte Wirksamkeit schließen. Bei Behandlung von Flusssedimenten mit 0,3 oder 3 g/L SDS wurde gezeigt, dass zwei Stunden nach Applikation nur noch 3,6% des Tensides nachweisbar waren (Seidel *et al*. 2000). Deshalb war nur bei der hohen Tensid- Konzentration eine kurzzeitige Hemmung der Mikroorganismen nachweisbar. Ähnliche Ergebnisse konnten in Perkolatoren im 63 m^3 Maßstab nach SDS Behandlung beobachtet werden (Schippers *et al*., 2001). Nach Zugabe von 30- 60 g/L SDS, was einer Dosis von 0,125 g SDS pro kg Haldenmaterial entsprach, konnte in der Laugungslösung nur noch bis zu 0,1 g/L SDS gemessen werden. Es wurde eine Verringerung der Stoffwechselaktivität im Haldenmaterial sowie eine Verringerung der Zellzahl, aber keine Abtötung der Laugungsorganismen gemessen. Durch eine Zugabe von 100 mg/L Isothiazolinon sowie Kalkstein und organischem Material zur Abdeckung wurde eine Reduzierung der Sulfat- oder Schwermetall- Freisetzung um etwa 50%, aber keine komplette Hemmung, erreicht (Sand *et al*., 2007).

1.4 Bakterielle Lyse

Der bakterielle Zelltod sowie die verschiedenen Möglichkeiten der Induktion der Zelllyse ist ein seit vielen Jahrzehnten bekannter und untersuchter Prozess (Rice und Bayles, 2008). Im Prozess des programmierten Zelltodes spielen neben anderen Faktoren auch Enzyme eine wichtige Rolle, wie Peptidoglucan Hydrolasen, sogenannte Autolysine (Höltje, 1995; Schockmann 1996). Diese Autolysine werden während des Wachstums in der Zellwand produziert und als inaktive Vorstufe in der Zellwand eingelagert (Rice und Bayles, 2008). Sie werden in der Zellteilung und Separation in Tochter- und Mutterzellen sowie in der Biofilmentwicklung aktiviert (Forsberg und Rodgers, 1971; Bayles, 2007). Für die Biofilmentwicklung ist der programmierte Zelltod nötig; dabei lysiert ein kleiner Teil der Population und setzt Makromoleküle wie Proteine und Nukleinsäuren frei (Steinmoen *et al*. 2002). Die freigesetzten Nukleinsäuren

können vom überlebenden, größeren Teil der Population aufgenommen werden (Kreth *et al*., 2009). Diese Nukleinsäuren sind weiterhin wichtig für die Struktur des Biofilms, für die Adhäsion der Mikroorganismen und wirken als Chelator von Kationen und vermitteln antibakterielle Aktivität (Allesen- Holm *et al*., 2006; Qin *et al*., 2007, Rice *et al*., 2007; Mulcahy *et al*., 2008, Das *et al*., 2009). Es ist bekannt, dass Laugungsbakterien, wie z.B. *A. ferrooxidans*, sowohl planktonisch als auch als Biofilm leben können. Über diesen vermitteln sie eine Bindung zum Substrat, z.B. Pyrit, und schaffen sich darin eine ideale Reaktionsumgebung (Sand und Gehrke, 2006).

Für eine Hemmung der Laugungsmikroorganismen in deren natürlichem Habitat und der damit zusammenhängenden behördlichen Genehmigung der Anwendung von Bioziden im Freilandversuch ist das Verständnis deren Wirkweise von großer Bedeutung. Die meisten Studien zum Mechanismus der Zelllyse wurden mit Modelorganismen wie *Escherichia coli*, *Bacillus subtilis* oder potentiell pathogenen Mikroorganismen wie *Staphylococcus epidermis*, *Streptococcus pneumonia*, *S. gordonii* oder *S. sanguinis* durchgeführt (z.B. Steinmoen *et al*., 2002; Kreth *et al*., 2009; Qin *et al*., 2007; Tsuchido *et al*., 1987; Cho *et al*., 1990; Liu und Burne, 2012). Dagegen gibt es nur wenige Untersuchungen zur Aufklärung der Lyse von Laugungsbakterien (Tuttle *et al*., 1977).

1.5 Zielstellung

In dieser Arbeit sollte der Nachweis von azidophilen Laugungsmikroorganismen im Lausitzer und Mitteldeutschen Braunkohlerevier erbracht werden. Ein Zusammenhang zwischen diesen Mikroorganismen und dem „Acid mine Drainage“ sollte gezeigt und eine Strategie zu dessen Hemmung entwickelt werden. Deshalb setzte sich die Arbeit aus vier Aufgaben zusammen.

Das erste Ziel dieser Arbeit war eine qualitative und quantitative Übersicht der azidophilen Mikroorganismen im Lausitzer und Mitteldeutschen Braunkohlerevier. Dafür wurden drei Lausitzer und zwei Tagebaue in Mitteldeutschland beprobt. Es sollten dabei aus einem Tagebau der Lausitz aus verschiedenen Schichten im Deckgebirge und der Kippenseite frisch freigelegte Proben sowie Proben für ein Altersprofil genommen werden. Weiterhin erfolgten Beprobungen an optisch auffälligen Stellen, z.B. an einem ölig, braunen Ausfluss oder an gelben Bodenverkrustungen. Aus allen fünf Tagebauen sollten zwei Jahre später aus verschieden Schichten des Deckgebirges sowie an optisch auffälligen Standorten Proben genommen werden. Diese Proben wurden durch die drei Methoden Kultivierung, Mikrokalorimetrie und molekularbiologische Untersuchung analysiert. Dabei sollte das Vorkommen der azidophilen Laugungsmikroorganismen, deren Stoffwechselaktivität, sowie die molekulare Charakterisierung der Spezies untersucht werden. Daraus sollte eine Gesamtaufnahme der vorhandenen Mikroorganismen erstellt werden, um zu wissen, welche Mikroorganismen im Teilprojekt drei und vier den Hemmversuchen unterzogen werden sollten.

Das zweite Ziel war herauszufinden, ab wann in der Entwicklung eines Tagebaus die Eisen- und Schwefel- oxidierenden Mikroorganismen beginnen aktiv zu werden. Für diese Fragestellung wurden Bohrkerne vor und nach Grundwasserabsenkung untersucht. Es sollten aus Sand, Schluff, Ton, Kohle sowie aus dem Liegenden Proben genommen und mit den oben genannten Methoden untersucht werden.

Als drittes Ziel sollte die Hemmung des AMD im Sand, dem natürlichen Habitat der Laugungsbakterien, untersucht werden. Dafür wurde in einem Lausitzer Tagebau ca. eine Tonne Material aus dem Deckgebirge genommen und anschießend mineralogisch sowie mikro- und molekularbiologisch

charakterisiert werden. Mit diesem Material waren Säulen in verschiedenen Größen zu befüllen, es sollte die Laugung gestartet werden um diese anschließend zu hemmen. Es sollten Hemmstoffe in verschiedenen Konzentrationen, in verschiedenen Temperaturbereichen, die unter anderem denen im natürlichen Habitat ähneln, getestet werden. Weiterhin sollte der Einfluss auf planktonische sowie im Biofilm lebende Mikroorganismen untersucht werden. Aus diesen Ergebnissen sollte eine Vorhersage für eine erfolgreiche Hemmung des AMD im Freiland- Versuch getroffen werden.

Das vierte Ziel war, den Wirkmechanismus von verschiedenen Oberflächen-aktiven Substanzen zu untersuchen, um die Hemmung des Bioleaching zu erklären. Dafür wurden planktonische Kulturen der Modelorganismen *E. coli* und *B. subtilis* sowie die bekannten Eisen- und Schwefel- oxidierenden Bakterien *A. ferrooxidans* und *A. thiooxidans* und der heterotrophe Mikroorganismus *Ac. cryptum* untersucht.
Eine Teilaufgabe stellte die Bestimmung der minimalen Hemmkonzentrationen der Tenside dar. Durch Messung der optischen Dichte bei 600nm (OD_{600}) für insgesamt 1000 Sekunden sollte quantitativ erfasst werden, ob die eingesetzten Tenside abtöten oder eine bakterielle Lyse induzieren. Da bei einer Zelllyse Makromoleküle freigesetzt werden, sollte der Gehalt von Proteinen oder Nukleinsäuren bestimmt sowie die Überlebensrate nach Tensid- Behandlung untersucht werden.

2. Ergebnisse und Diskussion

2.1 Erster Nachweis von Eisen- und Schwefel- oxidierenden Mikroorganismen in einem Lausitzer Tagebau in 2006

Für eine erste Bestandsaufnahme wurden im Jahr 2006 in einem aktiven Lausitzer Tagebau, 150 km südöstlich von Berlin, insgesamt 32 Proben aus dem unbeeinflussten Deckgebirge und von der Kippenseite mit einem Alter von bis zu 30 Jahren genommen. Zusätzlich wurden optisch auffällige Stellen, z.B. sichtbar durch braune Verfärbungen, beprobt.

In den Proben wurden durch Zellzählung mittels Fluoreszenzmikroskopie zwischen 10^2 und 10^7 Mikroorganismen pro Gramm Sand nachgewiesen. Dabei wurden die höchsten Zellzahlen im frisch freigelegten, unbeeinflussten Deckgebirge in der Haupt- und Grubenarbeitsebene gezählt, wogegen am Fuß des ersten Abwurfs der Kippe drei Größenordnungen weniger Mikroorganismen zu finden waren. In den Altersprofilen vom unbeeinflussten Deckgebirge und der Kippenseite wurden die geringsten Mikroorganismenzahlen gefunden. Die optisch auffälligen Proben zeigten im Durchschnitt die höchsten Zellzahlen zwischen $3x10^6$ und $2x10^7$ pro Gramm Material. Da Eisen- und Schwefel-oxidierende Mikroorganismen in einem Biofilm leben, ist davon auszugehen, dass zu geringe Zellzahlen bestimmt wurden, da es problematisch ist, Zellen aus einem Biofilm zu vereinzeln (Schippers *et al.*, 1995). Ähnliche Zellzahlen wurden in Uran Bergbauhalden in Deutschland und in der Höhle von Movile in Rumänien gefunden (Schippers *et al.*, 1995; Rohwerder *et al.*, 2003).

Durch Mikrokalorimetrie konnten in den Proben Gesamtaktivitäten zwischen einem µW und 69 µW pro Gramm Material gemessen werden. Die höchsten biologischen Aktivitäten mit bis zu 52 µW pro Gramm Sand wurden im frisch freigelegtem Deckgebirge gefunden, im Altersprofil der Kippenseite mit geringer Restfeuchte waren dagegen kaum biologische Aktivitäten messbar. Die meisten Proben bei denen pH- Werte unter fünf gemessen wurden, zeigten

biologische Aktivitäten über 5 µW pro Gramm Sand. Die höchste biologische Aktivität mit 52 µW pro Gramm Material wurde auf der Grubenarbeitsebene im frisch freigelegten Deckgebirge bei pH 2 und einer Restfeuchte von 4,8% gefunden. In einer 20 Jahre alten Probe aus dem Deckgebirge wurden bei vergleichbarem pH und Restfeuchte dagegen nur biologische Aktivitäten von 1,7 µW pro Gramm gemessen. Die hohe Aktivität von Proben mit hohen Zellzahlen von 10^6 bis 10^7 Zellen pro Gramm Material stimmt mit einer anderen Untersuchung überein (Schippers *et al*., 1995), dabei waren 10^3 bis 10^4 Zellen für eine Aktivität von 10 µW pro Gramm Material nötig.

Für den molekularbiologischen Nachweis von Eisen- und Schwefel-oxidierenden Mikroorganismen gelang es, aus 28 von 32 Proben Nukleinsäuren zu isolieren. Nach Aufreinigung von PCR- inhibierenden Substanzen wurden durch PCR aus 26 Proben bakterielle und aus vier Proben archaeale 16S rRNA Gene amplifiziert. Die Sequenzierung von zwei Archaea- spezifischen Amplifikaten zeigte eine 99 prozentige Übereinstimmung mit nicht kultivierten und nicht identifizierten Archaea aus der NCBI- Datenbank. Eine Nachbarschaftsanalyse gab Hinweise auf eine Verwandtschaft zu *Sulfolobus* spp., einem thermophilen Archaeon. Deshalb könnte man im Deckgebirge und der Kippe auf lokal begrenzte erhöhte Temperaturen von bis zu 80°C durch Pyritoxidation schließen, wie zuvor für Kupfererzhalden in den USA oder Kohlehalden in Deutschland beschrieben (Beck, 1967; Willscher *et al*., 2010). Durch Amplifikation der 16S rRNA Gene wurde in 23 Proben *A. ferrooxidans* und in 10 Proben *A. thiooxidans* nachgewiesen. Diese Amplifikation erfolgte nach Protokollen wie zuvor beschieben (De Wulf- Durand *et al*., 1997). Spezifische Amplifikate der 16S rRNA Gene vom Eisenoxidierer *L. ferrooxidans* wurden in neun Proben mit Primern nach Liu *et al*. (2006) nachgewiesen. Die Sequenzierung eines Teils der Amplifikate zeigte 96 bis 99% Übereinstimmung mit diesen drei Spezies. Diese Ergebnisse wurden durch eine erfolgreiche Isolation von 23 Reinkulturen mit der Doppel-Agar-Platten Technik

bestätigt (Johnson, 1995; Johnson *et al*., 2005). Sechs dieser Kulturen wurden nach Isolierung der Nukleinsäuren, Amplifikation und Sequenzierung der 16S rRNA Gene als *A. ferrooxidans* identifiziert. Der Nachweis von *Acidithiobacillus* spp. im Hauptteil der Proben, wogegen *L. ferrooxidans* nur in einem kleinen Teil der Proben nachgewiesen werde konnte, wurde auch für eine Kupfermine in Norwegen oder Halden in Botswana und Deutschland von anderen Arbeitsgruppen beschrieben (Johnson *et al*., 2001; Kock und Schippers, 2008).

In dieser ersten Bestandsaufnahme eines Lausitzer Tagebaus wurden also durch die vier unabhängigen Methoden Fluoreszenzmikroskopie, Mikrokalorimetrie, Kultivierung, Amplifikation und Sequenzierung der 16S rRNA Gene die bekannten Eisen- und Schwefeloxidierer *A. ferrooxidans*, *A. thiooxidans* und *L. ferrooxidans* nachgewiesen.

2.2 Nachweis erwarteter und unerwarteter Mikroorganismen im inzwischen fortgeschrittenen Tagebau im Jahr 2008

Zwei Jahre nach der ersten Bestandsaufnahme wurde im Jahr 2008 eine zweite Beprobung dieses Lausitzer Tagebaus durchgeführt. Dabei wurden an insgesamt zehn Stellen Proben genommen. Aus dem Deckgebirge in Höhe der Grubenarbeitsebene wurden vier Proben, auf Höhe der Hauptarbeitsebene wurden weitere zwei Proben genommen. Diese waren maximal eine Stunde der Luft ausgesetzt. Weiterhin wurden vier Proben an optisch auffälligen Stellen, wie an braunen Bodenverfärbungen oder an öligen Ausflüssen, genommen. Aus allen zehn Proben konnten nach Zhou *et al*. (1996) Nukleinsäuren extrahiert werden. Der Nachweis bakterieller 16S rRNA Gene gelang aus acht und der Nachweis archaealer 16S rRNA Gene aus zwei Proben. In neun Proben konnte *A. ferrooxidans*, in sieben Proben *A. thiooxidans*, jeweils durch Amplifikation der 16S rRNA Gene nachgewiesen werden, wogegen nur in einem Fall

spezifische Amplifikate für *Leptospirillum* spp. nachgewiesen wurden. Diese Resultate wurden durch erfolgreiche Kultivierung von mesophilen Eisen- und Schwefel- oxidierenden Mikroorganismen in allen Proben unterstützt. Aus den 32 Proben der ersten Probenahme in 2006 konnten aus 24 bzw. 27 Proben mesophile Eisen- oder Schwefel- oxidierende Mikroorganismen nachgewiesen werden. Die Kultivierung von moderat thermophilen Mikroorganismen bei 45°C gelang nur aus zwei Proben von 2008. Aus diesen Mischkulturen konnten Reinkulturen mit Hilfe der Doppel-Agar-Platten Technik nach Johnson *et al*. (2005), gewonnen werden. Nach Extraktion der DNA, Amplifikation der 16S rRNA Gene und Sequenzierung der Amplifikate konnten die Reinkulturen als *A. ferrooxidans, A. thiooxidans, A. albertensis, L. ferrooxidans, L. ferriphilum, Ferrimicrobium acidiphilum* und *Ac. acidophilum* identifiziert werden. Aus den beiden Mischkulturen bei 45°C wurden die isolierten Klone durch Sequenzierung der 16S rRNA Gene als *Sulfobacillus acidophilus* und *S. thermosulfidooxidans* identifiziert. Der molekularbiologische Nachweis der Eisen- und Schwefel- oxidierenden Bakterien und die Isolierung der neun Reinkulturen in dieser Arbeit bestätigten die Ergebnisse der Untersuchung des gleichen Tagebaus zwei Jahre zuvor.

Der Nachweis der erwarteten Eisen- und Schwefeloxidierer, wie z.B. *A. ferrooxidans,* kann mit den im Boden vorkommenden Temperaturen um 12°C und der Adaptierung von *Acidithiobacillus* spp. daran, sowie dem niedrigen pH Wert erklärt werden (Dopson *et al*., 2007; Kupka *et al*., 2007). Die erfolgreiche Reinkultur von *F. acidiphilum* aus dem Lausitzer Tagebau, zwei Jahre nach der Erstbeschreibung durch Johnson *et al*. (2009) mit einer optimalen Wachstumstemperatur um 35°C, sowie die Isolierung der beiden moderat thermophilen *S. acidophilus* und *S. thermosulfidooxidans* unterstützen die Hinweise auf Bereiche mit erhöhten Temperaturen. Die wiederholte Amplifikation von archaealer 16S rRNA Gene führte zu Kultivierungsversuchen bei erhöhten Temperaturen um 70°C, da Archaea als Besiedler extremer

Habitate bekannt sind. Die Kultivierungsversuche von Eisen- oder Schwefel-oxidierenden Mikroorganismen bei 70°C waren nicht erfolgreich.
Aber aus vier Proben wurden Reinkulturen isoliert, die durch Sequenzierung der 16S rRNA Gene als *Stenotrophomonas maltophilia, Acinetobacter calcoaceticus* und *Geobacillus caldoxylosilyticus* identifiziert wurden. Das war überraschend, da diese Bakterien als Bodenbesiedler bekannt sind, aber nicht für diese hohen Kultivierungstemperaturen beschrieben wurden (Palleroni und Bradbury, 1993; Duarte *et al*., 2001). *S. maltophilia* wurde aber beschrieben, Resistenzen, z.B. gegen Schwermetalle und Antibiotika, zu besitzen (Pages *et al*., 2009; Berg *et al*., 1999).

Zwei Jahre nach dem ersten Nachweis wurden die erwarteten Eisen- und Schwefel- oxidierenden Bakterien wieder durch klassische und moderne Methoden nachgewiesen. Die Isolierung der moderat thermophilen Bakterien und die Amplifikation archaealer 16S rRNA Gene können als Hinweis auf aufgeheizte Schichten im Deckgebirge gedeutet werden, hervorgerufen durch den Stoffwechsel der Mikroorganismen und chemische Oxidation des Pyrits.

2.3 Nachweise für Eisen- und Schwefel- oxidierende Mikroorganismen im Lausitzer und Mitteldeutschen Braunkohlerevier

Nachdem zwei unabhängige Untersuchungen in einem Lausitzer Tagebau zum Nachweis von Eisen- und Schwefel- oxidierenden Mikroorganismen geführt hatten, sollte nun das Vorkommen im gesamten Lausitzer und im Mitteldeutschen Braunkohlerevier untersucht werden. Im Jahr 2008 wurden verschiedene Probenahmen in den drei Tagebauen der Lausitz, sowie in zwei Tagebauen im Mitteldeutschen Braunkohlerevier durchgeführt. Es wurden 34 Proben an optisch auffälligen Stellen, am frisch freigelegten Deckgebirge, die

der Luft für maximal eine Stunde ausgesetzt waren und aus Sandschichten im Liegenden genommen.

Nach Extraktion der Nukleinsäuren direkt aus den Bodenproben und Aufreinigung von PCR- inhibierenden Substanzen wurden PCR Experimente zur Amplifikation der 16S rRNA Gene durchgeführt. 16S rRNA Gen Amplifikate der Eisen- und Schwefeloxidierer *A. ferrooxidans* oder *A. thiooxidans* waren in 14 bzw. 19 Proben zu finden, wogegen *Leptospirillum* spp. spezifische Amplifikate in acht Proben nachgewiesen wurden. Die erfolgreiche Kultivierung von Eisen- oder Schwefel- oxidierenden Mikroorganismen in 19 bzw. 20 Proben untermauert diese Ergebnisse. Es gelang weiterhin die Anzucht von insgesamt 168 Reinkulturen bei Kultivierungstemperaturen von 28 bis 45°C. Bei 28°C wurden sechs verschiedene Spezies durch Sequenzierung der 16S rRNA Gene identifiziert, *Acidithiobacillus ferrooxidans, A. thiooxidans, Leptospirillum ferrooxidans, L. ferriphilum*, *Acidiphilium acidophilum* und *Ferrimicrobium acidiphilum*. Bei Kultivierungstemperaturen von 45°C gelang die Isolierung und Identifizierung von *Sulfobacillus acidophilus*, *S. thermosulfidooxidans* und *S. thermotolerans*.

Diese Nachweise untermauern die Erkenntnisse aus den Untersuchungen von 2006 und 2008 im ersten Lausitzer Tagebau. Es wurden die erwarteten Eisen- und Schwefel- oxidierenden Mikroorganismen nachgewiesen (Rohwerder *et al*., 2003). Die Verteilung der *Acidithiobacillus* Spezies untereinander, sowie das Mengenverhältnis zu *Leptospirillum* spp. wurde in anderen Biotopen, z.B. aufgegebene Minen in Norwegen, AMD Gebieten in den USA und in Kohlehalden in Deutschland oder Botswana beschrieben (Johnson *et al*., 2001; Edwards *et al*., 1999; Kock und Schippers, 2008). Diese Verteilung könnte auf die Bodentemperaturen um 12°C zurückzuführen sein. Sie begünstigt die *Acidithiobacillus* spp. (Kupka *et al*., 2007) gegenüber den in Gebieten mit Temperaturen um 40°C beschriebenen *Leptospirillum* spp. (Edwards *et al*., 1999). Der wiederholte Nachweis von moderat thermophilen Bakterien mit

optimalen Wachstumsbedingungen bei Temperaturen zwischen 20 und 60°C und niedrigen pH- Werten um zwei (Bogdanova *et al*., 2006) lässt auf lokal begrenzte Bodenschichten mit erhöhten Temperaturen durch z.B. Pyritoxidation schließen, wie zuvor beschrieben (Beck, 1967; Willscher *et al*., 2010).

Analysen für Sulfat, Eisen und Sulfid von einem Lausitzer Tagebau zeigten geringe Sulfat- und hohe Eisen- Konzentrationen in frisch freigelegten Proben aus dem Deckgebirge. Mit der Erhöhung des Alters der Bodenproben auf bis zu 30 Jahre wurde eine Erhöhung der Sulfat- Konzentration und Verringerung der Eisenkonzentration in Proben aus dem Deckgebirge und von der Kippenseite gefunden. Diese Messungen wurden durch Analyse der Spurenelemente aus zwei Proben des frisch freigelegten Deckgebirges bzw. der 30 Jahre alten Kippenseite bestätigt.

Nach dem Nachweis der bekannten Eisen- und Schwefel- oxidierenden Mikroorganismen in den aktiven Braunkohle Tagebauen der Lausitz und in Mitteldeutschland sollte die Frage beantwortet werden, wann im Laufe der Vorbereitung, der Eröffnung und des fortlaufenden Betriebes eines Tagebaus die Laugungsmikroorganismen nachweisbar werden.
Dafür wurden 42 Proben aus vier Bohrungen nach Grundwasserabsenkung genommen. Aus 30 Proben konnten erfolgreich hochmolekulare Nukleinsäuren, größer als 10 kb extrahiert werden, sichtbar durch Anfärbung mit Ethidiumbromid nach Elektrophorese im Agarosegel. Amplifikation der bakteriellen 16S rRNA Gene gelang in 31 Fällen und archaeale 16S rRNA Gen-Amplifikate wurden in sechs Proben in Sandschichten gefunden. Die Eisen- und Schwefeloxidierer *A. ferrooxidans* und *A. thiooxidans* wurden durch Amplifikation der 16S rRNA Gene in 18 bzw. 21 Proben nachgewiesen. *Leptospirillum* spp. spezifische Amplifikate wurden in 11 Proben gefunden. Die Nachweise der bekannten Laugungsmikroorganismen in den Bohrkernen nach

der Grundwasserabsenkung unterstützen die Untersuchungen in den Tagebauen in der Lausitz und Mitteldeutschlands mit den Nachweisen der Eisen- und Schwefel- oxidierenden Bakterien. In mikrokalorimetrischen Untersuchungen der Proben waren mit maximalen biologischen Aktivitäten zwischen 29 und 310 µW pro Gramm Material ähnliche Werte wie in den Tagebauproben messbar und stimmen mit zuvor gezeigten Daten überein (Schippers *et al*., 1995). Die Kultivierung von Eisen- oder Schwefel- oxidierenden Mikroorganismen bei 28°C war dagegen nur in zwei bzw. 12 Proben erfolgreich. Diese Unterschiede zum molekularbiologischen Nachweis sind wahrscheinlich auf die Bohrtechnologie zurückzuführen, da die Bohrung mit einem Gemisch aus Wasser, Bentonit und Anisol gespült wurde. Diese Mischung könnte die Mikroorganismen inaktiviert oder gar abgetötet haben. Da genomisches Material im Boden, z.B. aus Pflanzen, noch nach mehreren Jahren nachweisbar ist (Widmer *et al*., 1997; Paget *et al*., 1998), wäre damit die höhere Nachweisrate in den PCR- Experimenten erklärbar.

Aus zwei Bohrungen vor Grundwasserabsenkung wurden 24 Proben genommen. Aus 16 Proben wurden erfolgreich Nukleinsäuren isoliert und bakterielle 16S rRNA Gene in nur fünf Proben amplifiziert. Amplifikate für die 16S rRNA Gene von Archaea, *A. ferrooxidans*, *A. thiooxidans* und *Leptospirillum* spp. waren nicht nachweisbar. Allerdings wurden aus zwei Proben spezifische Amplifikate für *Acidiphilium* spp. 16S rRNA Gene erzeugt. Kultivierungsexperimente bei 28°C gelangen mit einer Probe für Eisen- oxidierende und in acht Proben für Schwefel- oxidierende Mikroorganismen. In den mikrokalorimetrischen Untersuchungen wurden biologische Aktivitäten mit maximal 220 µW pro Gramm Boden gemessen.

Die Nachweise der Eisen- und Schwefel- oxidierenden Mikroorganismen aus den Bohrkernen vor und nach Grundwasserabsenkung, sowie die zuvor gezeigten Resultate, erlauben die Aufstellung einer Hypothese zum Start der

mikrobiellen Pyritoxidation. Die Eisen- und Schwefel- oxidierenden Mikroorganismen befinden sich im bergbaulich unbeeinflussten Deckgebirge in geringer Anzahl im Sand, weshalb nur in wenigen Proben der Nachweis durch Amplifikation der 16S rRNA Gene oder Kultivierung erfolgreich war. Durch die geringe Löslichkeit von Sauerstoff und Kohlendioxid im Grundwasser sind von diesen Gasen nur geringe Mengen verfügbar, wodurch die Mikroorganismen in einer Art Ruhezustand sind, wie z.B. in nährstoffarmen Habitaten beschrieben (Jones und Lennon, 2010). Mit der Absenkung des Grundwassers erfolgt ein Zustrom von Luftsauerstoff, die Lebensbedingungen für die Mikroorganismen verbessern sich und diese werden langsam aktiv und das Bioleaching beginnt. Durch die Verfügbarkeit von Sauerstoff in der Sand- oder Kohleschicht werden nur geringe Mengen vom Pyrit oxidiert. Mit dem Abbau der Braunkohle, dem Transport des Materials und der Vermischung auf der Kippe werden die aktiven Mikroorganismen im Material verteilt. Zu diesem Zeitpunkt ist Pyrit zusammen mit Sauerstoff und Kohlendioxid vollständig verfügbar, die Mikroorganismen sind vollständig aktiv und das Bioleaching ist maximal. Diese Hypothese wird auch durch die Untersuchungen zur Primäroxidation an der Abbaukante und auf der Kippenoberfläche unterstützt (van Berg und Wisotzky, 1995; Wisotzky, 1994; Wisotzky und Obermann, 2001). Die später in den rekultivierten Kippen stattfindende Sekundäroxidation wird dann durch die hohe Anzahl von Eisen- und Schwefeloxidierern beschleunigt. Mit fortschreitendem Alter der Kippe verringert sich dann die Verfügbarkeit des Pyrits. Dadurch verringert sich die Aktivität der Mikroorganismen, bis diese am Ende wieder in den Ruhezustand (Roszak und Collwell, 1987) übergehen.

2.4 Hemmung des Bioleaching durch Tenside in mit Sand gefüllten Säulen

Nach dem Nachweis der Eisen- und Schwefel- oxidierenden Mikrooorganismen im Lausitzer und Mitteldeutschen Braunkohlerevier sollten Strategien zur

Hemmung des Bioleachings entwickelt werden. Dafür wurden Versuche mit SDS und Texapon N70 in kleinen 20 mL und größeren 20 L Säulen durchgeführt. Diese Säulen waren mit Sand eines Grundwasserleiters aus einem Lausitzer Tagebau befüllt. Dieses Material war seit 13 Jahren entwässert und enthielt 0,4 bis 1% Pyrit. Die Experimente wurden in drei Temperaturbereichen von 21°C, 15°C bis 21°C und bei konstant 12°C durchgeführt.

Säulen im 20 mL Maßstab wurden mit ca. 16 Gramm Material befüllt. Dieses enthielt Eisen- oder Schwefel- oxidierende Mikroorganismen in Konzentrationen zwischen 1x 10^4 und 5x 10^4 Zellen pro Gramm Sand. Die Tensid- Behandlung der Säule, im ersten Lauf bei 21°C, erfolgte durch einmalige Zugabe von 85 mL Regenwasser mit 2 g/L SDS. Danach wurde nur noch mit Regenwasser gespült. Die Kontrollsäulen wurden entweder mit immer neuem Regenwasser beschickt oder der Durchlauf wurde wieder auf die Säule gegeben. Zu Beginn und am Ende des Experiments wurde die Konzentration der Eisen- und Schwefel- oxidierenden Mikroorganismen aus dem Boden und zusätzlich nach 13 Wochen vom Durchlauf mit der Most-Probable-Number Technik in zuvor beschriebenen Medien bestimmt (Mackintosh, 1978; Hutchinson *et al*., 1965).

Im ersten Lauf bei 21° C wurden in den Kontrollsäulen nach 26 Wochen etwa zwei Größenordnungen mehr Eisen- oxidierende Mikroorganismen pro Gramm Sand und zwischen 10^2 und 10^5 Schwefel- oxidierende Mikroorganismen pro Gramm Sand nachgewiesen. Im Sand der SDS behandelten Säule waren die Laugungsbakterien nach 26 Wochen nicht nachweisbar. Im Durchlauf der Kontrollsäulen waren nach 13 Wochen Eisen- und Schwefel- oxidierende Mikroorganismen in Konzentrationen zwischen 1x 10^4 und 5x 10^5 Zellen pro Milliliter nachzuweisen, in der SDS behandelten Säule dagegen nicht. Nach 26 Wochen waren im Durchlauf der SDS behandelten Säule Laugungsbakterien nicht nachweisbar, wogegen in den Kontrollen, welche nur mit Regenwasser behandelt wurden, 10^6 Zellen pro Milliliter nachweisbar waren. Die Abnahme

der Zellzahl stimmt mit zuvor gezeigten Arbeiten mit 1 mM SDS überein (Schippers *et al*., 1998). Wiederholte SDS Zugaben resultierten in einer dauerhaften Hemmung der Laugungsbakterien in großen Perkolatoren (Schippers *et al*., 2001). Die erfolgreiche Hemmung durch SDS wurde mit einer Eisen- Bestimmung des Durchlaufs unterstützt und zeigte 93% des Gesamteisen als Eisen (II), wogegen in den Kontrollen nur 1 bis 2% Eisen (II) nachweisbar war. Die Sulfat- Freisetzung wurde durch die einmalige SDS Zugabe um 25% verringert.

In weiteren Experimenten in den drei Temperaturbereichen von 21°C, 15 bis 21°C und bei konstant 12°C erfolgte die Tensid- Behandlung der Säulen durch einmalige Zugabe von 85 mL Regenwasser vermischt mit 4 g/L SDS, Texapon N70 oder einer 1:1 Mischung beider Tenside. Die Behandlung mit Regenwasser erfolgte wie oben beschrieben. Die Laugung in den Säulen wurde durch Zugabe von Regenwasser bei allen getesteten Temperaturen gestartet. Inhibition des AMD für eine Dauer von 38 Wochen wurde in den kleinen 20 mL Säulen bei allen Temperaturbereichen erhalten. Diese Ergebnisse wurden durch Wachstumsversuche mit den Durchläufen sowie den Bodenproben unterstützt. Diese zeigten am Ende des Experiments in den Tensid behandelten Säulen keine nachweisbaren Eisen- und Schwefel- Oxidierer, im Gegensatz zu den mit Regenwasser behandelten Proben mit Konzentrationen um die 10^6 Zellen pro Milliliter im Durchlauf oder 10^4 bis 10^6 Zellen pro Gramm Sand. Die Bestimmung von Eisen (II) und Gesamteisen im Durchlauf untermauerte diese Ergebnisse. In den Tensid behandelten Säulen wurde hauptsächlich Eisen (II) gefunden, was für eine erfolgreiche Hemmung der eisenoxidierenden Mikroorganismen spricht. In den mit Regenwasser behandelten Proben war die Laugung aktiv und deshalb wurde mit Eisen (III) hauptsächlich die oxidierte Form gemessen.

Für ein Upscaling wurden große 20 L Säulen mit ca. 12 bis 13 kg Sand befüllt. Dieses Material enthielt Eisen- oder Schwefel- oxidierende Mikroorganismen in

Konzentrationen zwischen 1x 10^4 und 5x 10^4 Zellen pro Gramm Sand. Die Säulen wurden in den Temperaturbereichen 15 bis 21°C oder bei konstant 12°C betrieben.

Durch wiederholte Zugabe von 2 oder 60 g/L Texapon N70 konnte eine erfolgreiche Hemmung des AMD für bis zu 45 Wochen im Sand und im Durchlauf der Säulen gezeigt werden. Die Sulfat- Freisetzung durch Texapon N70 und SDS Zugabe konnte dabei gegenüber der nur mit Regenwasser beladenen Säule um 29% verringert werden. Im ersten Lauf bei Behandlung mit 2 g/L Texapon N70 stieg die Konzentration der Eisen- und Schwefeloxidierer wieder an. Erklärungen dafür könnten in der Adsorption des Tensides am Sand, durch die natürliche Variabilität der Mikroorganismen hervorgerufene verringerte Sensitivität gegenüber des Texapon N70, oder im Anwachsen einer Texapon N70 abbauenden Mikroorganismen Population zu finden sein (Ying, 2006; Seidel *et al*., 2000; Sand *et al*., 2007). Nach der folgenden Zugabe von 10 g/L SDS waren keine Eisen- und Schwefel- oxidierenden Mikroorganismen mehr nachweisbar. Erfolgreiche Läufe mit 50 g/L Texapon N70, messbar durch Vorkommen von vorzugsweise Eisen (II) im Durchlauf der Säulen und Abtötung der Eisen- und Schwefel- oxidierenden Mikroorganismen, verdeutlichen die Notwendigkeit von ausreichend hohen Tensid-Konzentrationen am Wirkort, um die Mikroorganismen und damit das AMD über einen längeren Zeitraum zu hemmen (Seidel *et al*., 2000; Josza *et al*., 1999; Schippers *et al*., 2001; Schippers *et al*., 1998). In diesen Läufen wurde die kumulierte Sulfat- Freisetzung durch Texapon N70 Zugabe um 34 bis 44% verringert.

2.5 Tensid- induzierte Lyse von azidophilen Bakterien im Vergleich zu *E. coli* und *B. subtilis*

Um die erfolgreiche Hemmung des AMD durch Tenside in mit Sand befüllten Säulen erklären zu können, sollte die Wirkweise und der Einfluss auf

planktonische Zellen von Eisen- und Schwefel- oxidierenden Bakterien im Vergleich zu Modelorganismen untersucht werden.

Die Anwendung von Tensiden zur Hemmung der Eisen- und Schwefel-oxidierenden Mikroorganismen ist seit Jahrzehnten bekannt, die Ergebnisse variieren aber von Hemmung bis zu Anregung (Onysoko *et al*., 1984; Sand, 1985; Dugan, 1986). Deshalb sollte der Einfluss von Tensiden auf die Laugungsbakterien *A. ferrooxidans* ATCC 23270, *A. thiooxidans* DSM 622 und *Ac. cryptum* DSM 2389 sowie die Laborstämme *E. coli* und *B. subtilis* untersucht werden. Diese Untersuchungen wurden mit den anionischen Tensiden SDS und Texapon N70, einem Natriumlaurylethersulfat, von Cognis GmbH (Düsseldorf, Deutschland), durchgeführt.

Bei Wachstumsexperimenten mit planktonischen Laugungsbakterien wurden durch SDS oder Texapon N70 minimale Hemmkonzentrationen zwischen 0,005 und 0,05 g/L beobachtet, wenn das Tensid während des Wachstums zugegeben wurde. In der stationären Phase waren dagegen deutlich höhere Tensid-Konzentrationen von bis zu 2 g/L nötig um das Wachstum zu hemmen. Im Gegensatz dazu konnte beim Referenzorganismus *E. coli* bei diesen Tensid-Konzentrationen keine Hemmung beobachtet werden. Um diese Tensid-induzierten Effekte quantitativ messen zu können, wurde zu 10^{10} planktonischen Zellen von *A. thiooxidans* 0,5 bis 10 g/L SDS zugegeben. Nach 30 Minuten Inkubation wurde eine Abnahme der Zellen um 25 bis 75% gezählt. Eine Abnahme der Zellzahl, aber auch Überleben einer Restpopulation nach Autolyse wurde für *Staphylococcus epidermis* beschrieben (Qin *et al*., 2007). Um mehrere Proben schneller erfassen zu können, wurden Experimente im Photometer bei einer optischen Dichte von 600 nm (OD_{600}) mit konzentrierten Zellsuspensionen durchgeführt. Diese Suspensionen wurden auf eine OD_{600} von ca. 0,8 eingestellt. Im Anschluss wurden in den Photometerküvetten die Tenside in den Konzentrationen zwischen 0,001 und 10 g/L zugegeben und die Entwicklung der OD_{600} für 1000 Sekunden aufgezeichnet. Die drei Laugungsbakterien

zeigten nach 1000 Sekunden Tensid- Behandlung eine Abnahme der OD_{600} zwischen 50 und 80%. Behandlung der Referenzorganismen *E. coli* und *B. subtilis* mit bis zu 10 g/L Tensid zeigte dagegen nur einen leichten Abfall in der optischen Dichte bei 600 nm zwischen 10 und 20%. Der schnelle Abfall der OD_{600} um 50 bis 80% wurde als Hinweis auf eine Lyse der Laugungsorganismen gedeutet, wie für andere Mikroorganismen gezeigt wurde (Ensign und Wolfe, 1965; Leduc *et al*., 1982; Qin *et al*., 2007). Da bakterielle Lyse mit der Freisetzung von Makromolekülen wie Proteinen oder Nukleinsäuren verbunden ist, wurden diese Aspekte im Anschluss untersucht. Es konnte gezeigt werden, dass alle drei Laugungsorganismen, sowie die Referenzstämme Nukleinsäuren in Form von DNA und RNA nach Behandlung mit SDS oder Texapon N70 freisetzten. Die Freisetzung von Nukleinsäuren durch äußere Einflüsse wurde auch für andere Bakterien, wie z.B. *Streptococcus* spp. und *Enterococcus faecalis* beschrieben (Kreth *et al*., 2009; Das *et al*., 2010; Thomas *et al*., 2008). Diese Eigenschaft der Mikroorganismen ist wichtig in der Biofilm- Bildung, d.h. bei der Adhäsion der Zellen an das Substrat (Allesen-Holm *et al*., 2006; Das *et al*., 2009). Die Freisetzung von Nukleinsäuren nach Zugabe von organischen Säuren wurde für *A. ferrooxidans* beschrieben (Tuttle *et al*., 1977). Eine Freisetzung von Proteinen wurde dabei aber nicht gefunden. Dieser Aspekt sollte für die Laugungs- und Modelmikroorganismen untersucht werden. Nach SDS Zugabe konnte eine Freisetzung von 50 bis 80% der berechneten Gesamtproteine nur bei den Laugungsbakterien gemessen werden, für *E. coli* wurden im Maximum nur 0,35% der Gesamtproteine extrazellulär nachgewiesen. Die Unterschiede von *E. coli* zu den Laugungsbakterien mit Nukleinsäure- Freisetzung und fehlender Freisetzung von Proteinen könnte auf die Entwicklung von kompetenten *E. coli* Zellen hindeuten, wie für *Streptococcus pneumonia* beschrieben (Steinmoen *et al*., 2002). Diese Vermutung wurde durch Wachstumsexperimente nach Zugabe von 0,5 bis 10 g/L Tensid sowie durch Markierung mit Fluoreszenz- Farbstoffen untermauert.

Hierbei überlebten die *E. coli* Zellen, während die Laugungsbakterien abgetötet wurden.

Diese Ergebnisse deuten darauf hin, dass es eine ähnliche Wirkungsweise von SDS und Texapon N70 gibt. Die Referenz- oder Laugungsorganismen zeigten aber eine deutlich unterschiedliche Sensitivität bei Behandlung mit diesen Tensiden. Das könnte auf verschiedene Wirkmechanismen hindeuten, möglicherweise durch unterschiedliche Aktivierung oder Inaktivierung von Autolysinen. Die unterschiedliche Wirkung könnte aber auch an der höheren Wachstumsrate der Referenzorganismen und damit verbundener Ausbildung von Resistenzen gegen die Tenside begründet werden, was kompetente *E. coli* Zellen ermöglichen könnte. Bei *E. coli* und *B. subtilis* könnte die Tensid-Behandlung die Autolyse eines kleinen Teils der Population (Leduc *et al*., 1982; Cho *et al*., 1990; Tsuchido *et al*., 1985) durch Einbau der Tenside in die Zellmembran auslösen. Die Autolysine, beschrieben für *E. coli* (Höltje, 1995), werden dabei nicht oder nur teilweise aktiviert. Deshalb stirbt ein kleiner Teil der Population ab, wodurch nur ein kleiner Teil der Proteine messbar ist. Vom größeren, überlebenden Teil werden auch Nukleinsäuren abgegeben, diese sind aber noch an die Zellen gebunden. Diese Nukleinsäuren können dann von der überlebenden Gesamtpopulation aufgenommen oder zur Anheftung an Oberflächen genutzt werden (Steinmoen *et al*., 2002; Das *et al*., 2010).

Die Unterschiede der Laugungsbakterien in der Stressantwort auf die Tensid-Behandlung könnte auf Autolysin- ähnliche Enzyme zurückzuführen sein. Diese Enzyme wurden z.B. für *Streptococcus gordonii* beschrieben und waren an der Biofilmbildung, an der Säuretoleranz, sowie am Export von DNA beteiligt (Liu und Burne, 2011). Die Auswirkungen auf die Laugungsbakterien war von der Tensid- Konzentration abhängig. Bei hohen Tensid- Konzentrationen scheint eine schnelle Autolyse binnen weniger Sekunden aufzutreten, möglicherweise durch unspezifische Wechselwirkungen der Tensid- Moleküle in der

Zellmembran (Jones, 1999). Hierdurch wird die Zytoplasmamembran aufgelöst, wie z.B. für *E. coli* beschrieben (Filip *et al.* 1973).

Der zweite Weg bei geringen Konzentrationen scheint eine induzierte Zelllyse nur eines Teils der Population zu sein. Diese wird möglicherweise durch eine Teilaktivierung Autolysin- ähnlicher Moleküle in der bakteriellen Zellwand durch die Wechselwirkung mit nur wenigen Tensid- Molekülen ausgelöst. Für SDS wurde eine Änderung des Membranpotentials beschrieben (Arai *et al*. 1995), wodurch die Autolysine aktiviert werden (Bayles, 2007) und kleine Poren entstehen könnten. Diese wurden indirekt durch die Fluoreszenzfärbung der Zellen mit Propidiumiodid nachgewiesen und erlauben die Freisetzung von intrazellulären Proteinen. Diese Proteine könnten der Auslöser für die Freisetzung von Nukleinsäuren eines großen Teils der Population sein und könnten dann von einem Teil der Population aufgenommen (Steinmoen *et al*., 2002), oder als Auslöser für die Entwicklung eines Biofilms genutzt werden (Qin *et al*., 2007; Allesen- Holm *et al*., 2006; Rice, 2007). Bei geringeren Tensid- Konzentrationen sind die Poren möglicherweise so klein, dass nur kleine Signalpeptide freigesetzt werden, die für den Tot oder Hemmung eines Teils der Population, nicht aber für eine Autolyse sorgen, wie bei Wachstumsexperimenten in der stationären Phase gezeigt wurde. Für *S. pneumoniae* wurden Peptide des Zwei-Komponenten System VncR/S beschrieben, wodurch verschiedene Selbsttötungswege aktiviert werden (Novak *et al*., 2000).

2.6 Ausblick

In der vorliegenden Arbeit konnten erste Untersuchungen zum Wirkmechanismus der Tenside SDS und Texapon N70 durchgeführt werden und die praktische Anwendbarkeit zur Hemmung des AMD in mit Sand befüllten Säulen gezeigt werden. Aus diesen Resultaten können Ansatzpunkte für

zukünftige Arbeiten entwickelt werden, auf die in diesem Kapitel eingegangen wird.

In der Tensid- induzierten Zelllyse wurden deutliche Unterschiede zwischen *E. coli* einerseits und den drei Laugungsbakterien andererseits gefunden. *E. coli* reagierte in dem Klärungstest bei 600 nm langsamer und benötigte 10- mal höhere Tensid- Konzentrationen. Dazu passte die wesentlich geringere Proteinfreisetzung. Diese Befunde lassen darauf schließen, dass es unterschiedliche Mechanismen in der Auslösung der Zelllyse geben könnte. Deshalb wären die Aufklärung des Tensid- induzierten Lysemechanismus und der Wirkort des Tensides weitere Ansatzpunkte. Mit diesem Wissen könnten maßgeschneiderte Tenside entwickelt werden, die spezifisch gegen bestimmte Mikroorganismen wirken.

Anstatt nur die durch die Zelllyse freigesetzten Proteine zu messen, könnte auch die Freisetzung von Metaboliten, wie z.B. Citronensäure, untersucht werden. Weiterhin könnte der Einfluss von freigesetzten Signalpeptiden auf die Zelllyse sowie auf die Änderung des Anheftungsverhaltens der Mikroorganismen an das Substrat Gegenstand weiterer Untersuchungen sein.

Durch Tensid- Behandlung wurde bei allen untersuchten Bakterien eine Freisetzung von Nukleinsäuren gefunden. Hier stellt sich die Frage, ob die gesamte DNA ausläuft. Wenn das nicht der Fall ist, muss geprüft werden, ob statistisch alle Loci betroffen sind oder eine Bevorzugung oder Diskriminierung von Genen erfolgt. Der Einfluss der freigesetzten DNA auf die Biofilmbildung der Laugungsbakterien, z.B. an Pyrit, könnte ein Bestandteil weiterer Untersuchungen sein.

Da die Tensid- induzierte Nukleinsäurefreisetzung bei *E. coli* im Gegensatz zu den Laugungsbakterien nicht mit der Freisetzung von Proteinen sowie Zelllyse einherging, könnte die Ausbildung von Kompetenz eines Teils der Population mit folgender Ausbildung von Resistenzen einen weiteren Untersuchungspunkt darstellen.

Es könnten sich Experimente anschließen, in denen das Vorkommen und die Aktivität der autolytischen Enzyme untersucht werden. Bisher wurden diese Enzyme für die Laugungsorganismen noch nicht beschrieben. Durch eine Tensid- induzierte Änderung des Membranpotentials könnten diese Enzyme aus der inaktiven in eine aktive Form umgewandelt werden und dadurch die Lyse in Gang setzen.

Aus verschiedenen Bodenproben der Lausitzer und Mitteldeutschen Tagebaue wurden Amplifikate archaealer 16S rRNA Gene gewonnen. Diese wurden nach Sequenzierung und Abgleich mit NCBI- Datenbanken als nicht kultivierte und nicht identifizierte Archaea charakterisiert. Es wurde weiterhin durch Kultivierungsversuche bei 70°C ein Wachstum von Stäbchen beobachtet. Eine Identifizierung dieser Kultur gelang aber nicht. Weiterhin wurden durch Kultivierung bei 45°C verschiedene *Sulfobacillus* Spezies isoliert, weshalb auf das Vorkommen von Gebieten im Deckgebirge mit erhöhten Temperaturen geschlossen werden kann. Es ist davon auszugehen, dass in diesen durch chemische und mikrobielle Pyritoxidation aufgeheizten Habitaten eine Änderung der mikrobiellen Diversität im Gegensatz zu den bisher untersuchten Tagebau- Proben zu finden ist. Es könnte untersucht werden, ob sich diese „Hot spots“ im Deckgebirge nachweisen lassen und inwiefern die dort vorkommenden moderat Thermophilen Mikroorganismen eine vergleichbar hohe Sensitivität gegen Tenside besitzen wie die drei untersuchten Laugungsbakterien.

Diese Untersuchungen wären weiterhin für die Hemmung des AMD in einem Pilotversuch im Freiland wichtig, da gezeigt wurde, dass Eisen- und Schwefel-oxidierende Mikroorganismen vorzugsweise in Sand- und Kohleschichten vorkommen. Dadurch besitzen diese Schichten ein erhöhtes Risiko für eine AMD Bildung durch Pyritoxidation. Um die Applikation von Tensiden wie z.B. SDS oder Texapon N70 in einem wirtschaftlich vertretbaren Rahmen zu halten und die Umwelt nicht zu schädigen, wäre es zweckmäßig, die eingesetzte

Tensid- Konzentration zu minimieren oder nur in den Schichten mit hohem Risikopotential einzusetzen. Dafür wären Säulenversuche sinnvoll, welche mit an natürlich vorkommenden Wasser- und Temperaturverhältnissen durchgeführt werden.

Ein weiterer Punkt ist das Anheftungsverhalten der Laugungsmikroorganismen an Pyritminerale. Die Benetzung der Pyritmineralien mit den Tensiden könnte zu einer Veränderung des Oberflächenpotentials führen, wodurch eine spezifische Bindung der Laugungsbakterien an die Mineralien nicht mehr funktioniert.

Da die in der vorliegenden Arbeit eingesetzten Tenside SDS und Texapon N70 biologisch abbaubar sind, könnte untersucht werden, wie lange diese im Boden verfügbar sind und die Hemmung der Mikroorganismen anhält. Dafür müsste eine Analytik für die Tenside etabliert werden, um die im Boden und im Wasser verfügbare Tensid- Konzentration zu messen. Das würde Rückschlüsse auf die Adsorption der Tenside an die verschiedenen Bodenqualitäten ermöglichen.

Die Suche nach Tensid- abbauenden oder resistenten Mikroorganismen wäre ein weiterer Ansatzpunkt für zukünftige Arbeiten.

3. Literatur

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4. Veröffentlichungen und Manuskripte

4.1 Angaben zum Eigenanteil

Siebert, H.M., T. Rohwerder, W. Sand, M. Strzodka, and K.P. Stahmann (2009) Evidence for iron- and sulfur-oxidizing bacteria and archaea in a currently active lignite mining area of Lusatia (eastern Germany). Advanced Materials Research, 71/73:97-100

Konzeptentwicklung durch HMS, mikrobielle, molekularbiologische Analysen und mikrokalorimetrische Bestimmung durchgeführt von HMS, schreiben des Manuskriptes von HMS mit editorischer Hilfe der Co-Autoren.

Siebert, H.M., R. Marmulla, and K.P. Stahmann (2011) Effect of SDS on planctonic *Acidithiobacillus thiooxidans* and bioleaching of sand samples. Minerals Engineering, 24(11):1128-1131

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Konzeptentwicklung durch HMS, mikrobielle Analysen durchgeführt von HMS, TU, SH, schreiben des Manuskriptes von HMS mit editorischer Hilfe von KPS und WS.

Siebert, H.M., B. Florian, J. Havenstein, S. Krüger, W. Sand, and K.P. Stahmann (2012). Iron- and sulfur- oxidizing microorganisms in drilling cores, overburden and heaps of five lignite mining areas in Germany.
Das Manuskript wurde bei AEM eingereicht.
Konzeptentwicklung durch HMS, mikrobielle und molekularbiologische Analysen durchgeführt von HMS, JH, SK, mikrokalorimetrische Bestimmung durchgeführt von BF, schreiben des Manuskriptes von HMS mit editorischer Hilfe von KPS und WS. HMS ist korrespondierender Autor.

Siebert, H.M., Y. Wloka, and K.P. Stahmann (2012) Cell lysis of leaching bacteria explains inhibition of pyrite oxidation observed after treatment of sand samples with surfactants.
Das Manuskript wurde bei AEM eingereicht.
Konzeptentwicklung durch HMS, mikrobielle und molekularbiologische Analysen durchgeführt von HMS, YW, schreiben des Manuskriptes von HMS mit editorischer Hilfe von KPS und WS. HMS ist korrespondierender Autor.

4.2 Veröffentlichungen

4.2.1 Evidence for iron- and sulfur-oxidizing bacteria and archaea in a currently active lignite mining area of Lusatia (eastern Germany)

Siebert, H.M., T. Rohwerder, W. Sand, M. Strzodka, and K.P. Stahmann (2009) Advanced Materials Research, 71/73:97-100

Advanced Materials Research Vols. 71-73 (2009) pp 97-100
online at http://www.scientific.net

Online available since 2009/May/19

Evidence for iron- and sulfur-oxidizing bacteria and archaea in a currently active lignite mining area of Lusatia (eastern Germany)

H.-M. Siebert[1a], T. Rohwerder[2b], W. Sand[2c], M. Strzodka[3d] and K.-P. Stahmann[1e]

[1]Bio-, Chemie- und Verfahrenstechnik, Hochschule Lausitz, Großenhainer Str. 57, D-01968 Senftenberg, Germany

[2]Aquatische Biotechnologie, Biofilm Centre, Fachbereich Chemie, Universität Duisburg-Essen, Geibelstr. 41, D-47057 Duisburg, Germany

[3]Gesellschaft für Montan- und Bautechnik mbH (GMB), Knappenstr. 1, D-01968 Senftenberg, Germany

[a]hsiebert@fh-lausitz.de, [b]thore.rohwerder@uni-due.de, [c]wolfgang.sand@uni-due.de, [d]m.strzodka@gmbmbh.de, [e]stahmann@fh-lausitz.de

Keywords: Lignite mining, Microcalorimetry, 16S rRNA gene, PCR

Abstract. The largest lignite mining area in Europe is located 150 km southeast of Berlin. Acidic lakes exist in this area, known to be caused by marcasite oxidation. Thirty-two samples from the open-pit brown coal-mine Jaenschwalde were analyzed for microorganisms. Cell numbers determined after separation from sand particles revealed concentrations of 10^2 to 10^7 microorganisms per g sample. In samples exposed to the air within an hour, up to $4x10^7$ cells were counted. Measurement of metabolic activity by microcalorimetry showed for such samples up to 50 µW per g sand, whereas in heap samples (with low moisture) low or even no activity was measurable. DNA extraction was successful for 28 samples. In 26 samples microbial 16S rRNA genes were amplified by PCR. *Acidithiobacillus ferrooxidans* and *At. thiooxidans* specific amplificates were detected by nested PCR in 23 and 10 cases, respectively. A specific signal indicating *Leptospirillum ferrooxidans* was obtained with nine samples. Random samples were sequenced and showed 96 to 99 % identity with published data of all three species. Surprisingly, in four samples archaeal 16S rRNA genes were amplified by PCR. Sequencing of two samples showed 99 % identity with unidentified or uncultured archaea found in NCBI-databases. Molecular biology results for *At. ferrooxidans* as well as for *At. thiooxidans* were supported by successful isolations of pure cultures in 23 cases. Cultivation of the archaea failed so far. These data indicate that iron- and sulfur-oxidizing microorganisms occur at these sites in large numbers. If in addition the evidence for archaea can become verified, a screening for hot spots as the sites of their occurrence would become interesting.

Introduction

Exclusively marcasite occurs in Lusatia together with brown coal and overburden. After lowering the groundwater and mining of the brown coal, reduced sulfur components have been oxidized. The oxidation of marcasite leads with water to sulfuric acid containing products. This is known as acid mine drainage (AMD).

Open brown coal mining area of Jaenschwalde is located northeast of Cottbus close to the borderline to Poland. Lowering of groundwater level started in 1972 and open-pit mining in 1976. Since opening around 513 million tons brown coal with a heat value of 8 MJ per kg were mined.

In this mining landscape metal sulfide oxidizing microorganisms, extremely acidophilic bacteria, occur such as the mesophilic, chemolithoautotrophic iron and/or sulfur oxidizing bacteria *At. ferrooxidans*, *At. thiooxidans* and *L. ferrooxidans*. Ground water temperatures around 10 °C

throughout the year make the occurrence of moderate thermophilic pyrite oxidizing bacteria, and/or *Sulfolobales*, an order of extreme thermophilic sulfur- and iron(II)-oxidizing archaea, unlikely.
The goal of this work was a qualitative and quantitative detection of iron- and sulfur-oxidizing microorganisms in this area. Two profiles were investigated, first a comparison of fresh samples from different depths, especially from tertiary strata, in overburden or heap, second an age profile of overburden and heap (one month up to 30 years). Furthermore, six samples at optically striking sites were analyzed. Further investigations clarified, whether bacterial or archaeal species, known to exist in AMD area, are experimentally accessible and detectable by molecular biological methods.

Material and Methods

Sampling. Thirty-two samples from the mine Jaenschwalde were collected. This mining area, exploited by Vattenfall Europe Mining AG, encompasses waste heaps up to 30 years old that are both, accessible and fully documented. Two sampling campaigns, in April and July 2006, took place. The upper 30 to 50 cm dry sand layer was removed and about 250 g sample was taken. Mapping of the samples was performed by GPS (global positioning system). Ten samples were taken from fresh overburden upper and middle level and foot of the first release of the heap area, exposed to the air within an hour. Furthermore two age profiles, with 16 samples, were carried out with an age of one and six months, one, two, five, ten, 20 and 30 years, from the accessible overburden and the edge hose of the heap side. Furthermore six striking samples were taken, represented for example by orange-brown discoloring of the sand.

Cell Counting. Cells were counted in a Thoma chamber following a washing procedure for the samples to separate microbes from solid, high-density particles which would interfere with microscopy. To distinguish between particles and microorganisms, DNA was stained with 4´,6-diamidino-2-phenylindole (DAPI). From each sample three light microscope and fluorescence pictures were captured (Axioscope 2 plus, Axiocam HRC, Zeiss, Germany) and counted.

Metabolic Activity. Microcalorimetric investigations were performed as two-step measurements [1]. The first measurement with the untreated sample was carried out to determine the entire activity i.e. biological and chemical activity. After heat inactivation of mesophilic microorganisms at 60 °C, the remaining activity of the sample was measured again. The difference of the two measurements resulted in the energy delivered by microbial metabolism. For determination of dry weight, samples were stored open in the drying chamber for two to four days at 60 °C.

Molecular Biological Experiments. DNA extraction from 7.5 g sample was performed as previously described [2] and concentrations were visualized by staining with ethidium bromide after electrophoresis on 0.8 % (w/v) agarose gels. Extracted DNA was further purified from humic substances and PCR interfering substances by PowerClean DNA Clean-up Kit (MoBio Laboratories Inc., United States). DNA from cultures was isolated with another protocol [3]. For detection of lithotrophic microorganisms four PCR protocols were used. With the first protocol bacterial 16S rRNA genes were amplified, as described [4] and a 1.5 kb product was achieved. PCRs with specific primer pairs for *At. ferrooxidans* and *At. thiooxidans* were carried out as nested PCR with the 1:10 diluted PCR product of bacterial 16S rRNA gene amplification [5]. *L. ferrooxidans* was detected with a specific and universal primer [3]. 50 µl reaction mixture consisted of PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP Mix, 0.5 µM of each primer, one unit *Taq* DNA polymerase (Jena Bioscience, Germany) and 0.1 to 1 ng of purified DNA. Archaeal 16S rRNA genes were amplified with 2 mM $MgCl_2$ and additional 5 % DMSO [6,7].

Cultivation. Pure cultures of iron- and sulfur-oxidizing bacteria were obtained by inoculation with samples after washing procedure and cultivation [8]. Acidophilic bacteria were purified by repeated isolation of single colonies. Plates were incubated at 28 °C for four weeks. For maintenance of iron- or sulfur-oxidizing bacteria medium containing 10 g/L ferrous iron, or 10 g/L elementary sulfur was used [9,10]. Cultivation of thermophilic microorganisms was performed in test tubes at 45, 55 or 70

°C with liquid media (DSMZ designations) 88 (*Sulfolobus*), 665 (*Sulfobacillus*), 150 (*Ac. brierleyi*) and 182a (*S. solfataricus*).

Results and Discussion

Microorganisms occurred in a range from 10^2 to 10^7 cells per g sand (Table 1). Freshly exposed samples exhibited maximal cell counts for the upper level and for the middle level. Fresh samples of the heap had shown down to three orders of magnitudes less cells per gram sand. Age profile samples with an age up to 30 years showed lowest cell counts for grown acclivity as well as for heap samples. Striking samples, e.g. by orange-brown color, showed in average the highest values between $3x10^6$ and $2x10^7$ cells per gram sand. Obviously there is heterogeneity in sand composition, water content and cell count. The real cell number was most probably underestimated, because microorganisms growing as a biofilm cannot be separated into single cells [1]. Similar cell numbers per g sand were described for uranium mine waste heaps and Movile Cave, respectively [1,11]. Investigations were carried out to quantify metabolic activity of microorganisms by microcalorimetry. By this measurement, heat production was shown in all samples with ranges between 1 µW per g and 69 µW per g dry sand. In a fresh overburden sample up to 50 µW per g sand was measured, whereas in heap samples with low moisture little or no activity was determined. Samples with pH values lower than 5 showed in most cases biological activity of more than 5 µW per g sand. The highest biological activity occurred in a sample of middle layer with 52 µW per g sand. This sample had a pH of 2 and a water content of 4.8 %. Low biological activity was found in the 20 year old material with 1.7 µW per g sand at comparable pH and water content. Unexpected were the high biological activities in the fresh overburden samples, therefore a closer look to humidity of the samples was taken. Fresh samples from overburden and heap showed values between 2 % and 9 % water. Furthermore, decreasing water content down to 0.5 % in the age profile samples was determined. The striking samples showed values around 25 % water. The cell counts of samples with high heat output, i.e. 10^6 to 10^7 cells per g sand, agreed with data published by Schippers et al. [1]. There 10^3 to 10^4 cells from laboratory or environmental samples were needed for a heat output of 10 µW per g ore.

Sample age	origin	Cells [per g]	Activity [µW / g]	*At. ferrooxidans* [PCR + / N]	*At. thiooxidans* [PCR + / N]	*L. ferrooxidans* [PCR + / N]	*Archaea* [PCR + / N]
1h	O	10^5 - 10^7	1.6 - 52	4 / 6	3 / 6	1 / 6	0 / 6
	H	10^4 - 10^6	3.8 - 14	3 / 4	4 / 4	2 / 4	0 / 4
1m-30y	O	10^2 - 10^5	0.3 - 4.0	7 / 8	0 / 8	2 / 8	3 / 8
	H	10^2 - 10^6	0.1 - 4.6	6 / 8	1 / 8	1 / 8	1 / 8
Striking		10^6 - 10^7	1.7 - 30	3 / 6	2 / 6	3 / 6	0 / 6

Table 1 Number of microbial cells, metabolic activity, and PCR signals (+) for three species plus archaea in 32 samples (N) taken from overburden (O) or heap (H); age profile from 1 hour (h) over 1 month (m) up to 30 years (y) plus samples striking e.g. by orange-brown color.

The samples were investigated for leaching microorganisms by DNA extraction, purification and amplification by different PCR protocols. Extraction of DNA was carried out once. Genomic DNA larger than 23 kb was detected in 28 of 32 samples. In 26 samples microbial 16S rRNA genes were amplified. Signals specific for *At. ferrooxidans* were detected in 23 of 32 samples and for *At. thiooxidans* in 10 of 32. The iron- and sulfur-oxidizing bacterium *At. ferrooxidans* was the dominating microorganism, independent of sampling site or age of the samples (exposition time to additional atmospheric oxygen). Only in 10 and 20 years old samples *At. ferrooxidans* was not detectable. The sulfur-oxidizing bacterium *At. thiooxidans* was found in seven of ten fresh samples. In age profiles it occurred only occasionally. The iron-oxidizing bacterium *L. ferrooxidans* was found sporadically in nine samples. Random samples were sequenced and showed 96 to 99 % identity with published data of all three species. Dominance of *At. ferrooxidans* was as expected

due to temperature and humidity values. In Lusatia lignite mining area temperature means from 1961 to 1990 were recorded between -0.8 °C and 18.4 °C [12]. These temperatures are more conducive to the growth of some strains of *At. ferrooxidans* than *L. ferrooxidans* [13]. Successful isolation of pure cultures in 23 cases and identification by sequencing, of six clones, as *At. ferrooxidans* support these findings.
Archaeal 16S rRNA genes were detected in various samples. Sequencing of two samples showed 99 % identity with unidentified or uncultured archaea found in NCBI-databases. The next neighbor analysis of the amplificates gave evidence for thermophilic microorganisms related to *Sulfolobus* spp. and suggests the existence of hot spots with up to 80°C in the overburden of mining environment [14]. This temperature was shown to be caused by oxidation of sulfidic ores, sufficient to maintain large heaps above 70 °C [3,15]. The relatively high biological activity with up to 50 µW per g sand even after inactivation of mesophilic microorganisms at 60 °C may also indicate the presence of thermophilic or hyperthermophilic microorganisms. Samples were also investigated for the presence of *At. caldus, Sulfolobus* spp., *S. acidophilus* and *S. thermosulfidooxidans*. These microorganisms were not detected in any sample. Growth experiments with media and temperatures, according to the conditions known for the next neighbors from sequencing analysis, were performed. Cultivation of the archaea failed so far, but with medium 182a, growth at 70 °C was visible. Microscopic examination and amplification of 16S rRNA genes revealed bacterial growth. Characterization of this mixed culture is performed. In future, leaching experiments including inhibitor applications are planned to test for AMD countermeasures.

Conclusions

By four independent experimental approaches, direct fluorescence microscopy, microcalorimetry, cultivation, 16S rRNA gene amplification and sequencing, *At. ferrooxidans*, *At. thiooxidans*, *L. ferrooxidans* and two archaea were identified in an active lignite site in Lusatia.

Acknowledgements. We are grateful to Gesellschaft für Montan und Bautechnik (GMB) for financial support.

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Biohydrometallurgy 2009
doi:10.4028/www.scientific.net/AMR.71-73

Evidence for Iron- and Sulfur-Oxidizing Bacteria and Archaea in a Currently Active Lignite Mining Area of Lusatia (Eastern Germany)
doi:10.4028/www.scientific.net/AMR.71-73.97

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4.2.2 A Lusatian open cast lignite mine - a source of new iron-oxidizing microorganisms?

Siebert, H.M., T. Uhlig, S. Hondke, W. Sand, and K.P. Stahmann (2011) Proceedings of the 19th International Biohydrometallurgy Symposium, Changsha, China. G. Qiu, T. Jiang, W. Qin, X. Liu, Y. Yang, H. Wang (eds.), ISBN 978-7-5487-0356-3, 1002-1006

A lusatian open cast lignite mine—A source for new iron-oxidizing microorganisms?

Hans-Michael Siebert[1,a], Tobias Uhlig[1,b], Silvia Hondke[1,c], Wolfgang Sand[2,d], K. Peter Stahmann[1,e]

[1] Bio-, Chemie-und Verfahrenstechnik, Hochschule Lausitz (FH), Großenhainer Str. 57, D-01968 Senftenberg, Germany

[2] Aquatische Biotechnologie, Biofilm Centre, Fakultät für Chemie, Universität Duisburg-Essen, Universitätsstraße 2, D-45141 Essen, Germany

[a] Hans-Michael. Siebert@HS-Lausitz. de, [b] tobuhl@gmx. de, [c] sylvia_hondke@gmx. de, [d] wolfgang. sand@uni-due. de, [e] Klaus-Peter. Stahmann@HS-Lausitz. de

Keywords: acid mine drainage; bioleaching; lignite mining; 16S rRNA gene

Abstract: Acid mine drainage (AMD) with high sulphate concentrations triggered a microbiological study in an open lignite mining area in Eastern Germany as a basis for a development of sustainable countermeasures [1]. In 2006 and 2008 sampling campaigns were performed.

In 2006 the DNA of *Acidithiobacillus ferrooxidans*, *A. thiooxidans*, and *Leptospirillum ferrooxidans* was detected by PCR experiments. The *Acidithiobacillus* species were equally distributed in the overburden.

In the 2008 campaign four sand samples were taken ten meters above the lignite, two samples from aquifer material, and four samples visibly striking by brown colour.

All samples allowed DNA extraction. Bacterial 16S rRNA genes and in the two samples from aquifer material archaeal 16S rRNA genes were amplified. Specific amplificates for *A. ferrooxidans* and *A. thiooxidans* were achieved from nine or seven samples, respectively. In one aquifer material sample an amplificate specific for *Leptospirillum* spp. was detected.

Pure cultures at 28℃ were achieved for *A. ferrooxidans*, *A. thiooxidans*, *L. ferrooxidans*, *L. ferriphilium* and *Acidiphilium acidophilium*. Surprisingly, *Ferrimicrobium acidiphilium*, a bacterium described in 2009 for the first time [2], also occurred in aquifer material from the fresh overburden. Cultivation experiments for iron- and sulphur-oxidizing microorganisms at 28℃ with the 2008 samples revealed growth in all cases. Furthermore, by cultivation at 45℃ iron-oxidizing microorganisms were detected in two aquifer samples from 2008, whereas in 2006 no such activity was traceable. One pure culture was achieved and identified by sequencing of the 16S rRNA genes as *Sulfobacillus acidophilus* with 99 percent identity.

The detection of *F. acidiphilium* clearly encourages the search for other unknown leaching microorganisms in these acid mine drainage areas.

To abate AMD first experiments with tensides like SDS are performed. A serious reduction of sulphate efflux from sand samples with up to 1% of pyrite as source of acidification and sulphate was achieved.

1 Introduction

Acid mine drainage is a worldwide problem in mining associated regions. In the mining area of Lusatia iron sulphides e. g. pyrite and marcasite, were found in the brown-coal layer and overburden. The oxidation of these sulphide containing minerals leads to sulphuric acid containing products. The dissolved metals contaminate ground and surface water, a phenomenon known as acid mine drainage (AMD). Extremely acidophilic, metal sulphide oxidizing microorganisms mediate and accelerate this process. The process of leaching by bacteria has been widely studied. The mechanisms as well as the involved microorganisms were reviewed [3].

In 2006 the acidophilic iron- and sulphur-oxidizing bacteria *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans* were identified in a study of an East German lignite mining area. In the overburden *A. ferrooxidans* and *A. thiooxidans* were equally distributed, in heap samples *A. ferrooxidans* was the dominant acidophile [1].

To protect the environment from AMD the inhibition of bacterial and chemical leaching is necessary. The application of tensides like sodium dodecyl sulphate (SDS), resulted in inhibition or enhancement [4-6]. The efficient inhibition of leaching bacteria by SDS application and introduction of alkaline layers in large percolators was also described [7]. The inhibitory effect can be explained by alysis of the cells, shown by the release of nucleic acids [8].

The goal of this work was a qualitative detection of

iron-and sulphur-oxidizing microorganisms in this area two years after the first sampling campaign in 2006. Two different sample series were taken, six systematic samples from tertiary strata in the overburden, exposed to the air up to one hour. Furthermore four samples at optically striking sites. Further investigations clarified, whether bacterial or archaeal species, known to exist in AMD area, are experimentally accessible and detectable by molecular biological methods. This analysis allows the preparation of a microorganisms database relevant for microbial leaching in the Lusatian mining area, to develop methods for the specific inhibition of these microorganisms.

2 Material and methods

2.1 Sampling

Two years after the sampling campaign, described in 2009 [1], further ten samples were taken in the same open lignite mining area (51°80′N; 14°50′E) in August 2008. All samples were from tertiary strata and grouped into systematic and striking samples. Four of the systematic samples were taken ten meters above the lignite and additional two samples at a higher level. These six systematic samples were not more than one hour exposed to the air, before 250 g of sand were taken. The four other samples were taken at optically striking sites, e.g. brown colouring of the sand, or on oily effluent in the lignite mining area.

2.2 Cultivation and isolation of pure cultures

Samples were cultured in test tubes with medium containing 10 g/L ferrous iron, or 10 g/L elementary sulphur at 28℃ or 45℃ [9, 10]. Cultivation at 70℃ was performed with the two media, supplemented with 0.1 to 0.5 percent yeast extract, or with 9K- Medium and 10 g/L ferrous sulphate, supplemented with or without 2.5 mM potassium tetrathionate [11]. Acidophilic bacteria were purified by repeated isolation of single colonies with double-agar plate technique [12]. Plates were incubated at 28℃ for up to four weeks. For isolation of bacteria at temperatures above 45℃ plates with media supplemented with 0.8 percent gellan gum were used.

2.3 Molecular biological methods

Extraction of nucleic acids from 7.5 g of the sand samples was performed as previously described [13]. The purification from humic or other PCR interfering substances was performed by PowerClean™ DNA Clean-up Kit (No. 12877 – 50, MoBio Laboratories Inc., United States) according to manufactures instructions. The extraction of nucleic acids from liquid cultures or from single colonies picked from the agar plates was performed with another protocol [14].

The bacterial orarchaeal 16S rRNA genes were amplified as described [15, 17]. The amplification of the 16S rRNA genes of leaching associated bacteria was performed with protocols described before [14, 18].

Sequencing was performed with all PCR amplificates from pure cultures or picked clones and random amplificates of species specific PCR after purification with the Nucleo Spin® extract II (No. 740.609.50, Machery-Nagel GmbH & Co. KG, Germany) according to manufacturer's instructions.

3 Results and discussion

From the ten sand samples of the sampling campaign in 2008 the extraction of nucleic acids was performed twice and successful in all cases, visible by a size above 23 kb. After purification from humic acids and other PCR interfering substances, amplification for the bacterial 16S rRNA gene was performed with two different protocols and successful for all six systematic samples, and two of the striking samples (Table 1.). Amplificates for the archaeal 16S rRNA gene were detected in two of the systematic samples, but not in the striking ones. Sequencing of the 16S rRNA gene amplificates showed 97 to 98 percent identity with uncultured crenarchaeota found in NCBI databases. This is similar to our observations described two years earlier [1]. The iron- and sulphur-oxidizing bacteria *A. ferrooxidans* and *A. thiooxidans* were detected in all systematic samples, whereas in the striking samples only three or one amplificate was achieved. *Acidithiobacillus* species were found as the majority in all samples, whereas only in one sample from the aquifer material an amplificate for *Leptospirillum* spp. was detected. This support also the results shown before, and might be explainable by temperature or humidity optima. To answer the question whether these amplificates resulted also in the detection of living and metabolic active iron- and sulphur-oxidizing microorganisms, cultivation experiments were performed in a temperature range from 28℃ to 70℃, for mesophilic to thermophilic microorganisms.

At 28℃ growth of iron-oxidizing microorganisms, in media containing 10 g/L ferrous iron, was found in all ten samples. In all samples from 2008 also the growth of mesophilic sulphur-oxidizing microorganisms was visible.

For isolation of pure cultures and subsequent identification by sequencing of the 16S rRNA genes the double-agar plate technique was used. For a fast overview above the composition of the cultures, clones were picked from the plates, DNA was extracted and the 16S rRNA genes were amplified and sequenced. Analyses of the picked clones showed for the systematic samples from the aquifer material, a distribution of the leaching bacteria. In these samples the *Acidithiobacillus* species including *A. ferrooxidans*, *A. thiooxidans*, and *A. albertensis* as well as *Ferrimicrobium* species with an

Table 1. Molecular biological based detection of iron- and sulphur-oxidizing microorganisms from sampling campaigns in 2006 and 2008 (51°80′N; 14°50′E). Detection occurred after direct DNA isolation by PCR experiments as described [14 – 18]. Amplificates of 16S rRNA genes specific for bacteria, archaea, as well as for the species *A. ferrooxidans* *A. thiooxidans*, and *Leptospirillum* spp.. Listed are the ratios of positive samples/total samples.

Detection by amplification of 16S rRNA gene						
Sample origin		Bacteria	Archaea	*A. ferrooxidans*	*A. thiooxidans*	*Leptospirillum* spp.
2006	systematic	23/26	4/26	20/26	8/26	6/26
	striking	3/6	0/6	3/6	2/6	3/6
2008	systematic	6/6	2/6	6/6	6/6	1/6
	striking	2/4	0/4	3/4	1/4	0/6

identity between 99 and 100 percent identity were found. From the striking samples the picked clones were identified with 97 to 100 percent as *A. ferrooxidans*.

Sequencing of the DNA amplificates from pure cultures after repeated isolation showed similar results. In samples from aquifer material a mixture of *Acidithiobacillus* species and *Ferrimicrobium* species were isolated and identified with 99 to 100 percent identity, whereas in the striking samples only the iron- and sulphur-oxidizing bacterium *A. ferrooxidans* was detected with 97 to 100 percent identity (Table 2). Additionally, from the 32 sand samples taken 2006 pure cultures of the leaching bacteria were achieved by cultivation at 28℃ and isolation by this technique. These iron- and sulfur-oxidizing bacteria were identified after DNA extraction and sequencing of the amplified 16S rRNA genes, as *A. ferrooxidans*, *A. thiooxidans*, *L. ferrooxidans*, *L. ferriphilium*, *Ferrimicrobium acidiphilium* and *Acidiphilium acidophilium* with 98 to 100 percent identity.

The detection and isolation of the expected leaching bacteria can be explained by the low pH and overburden temperatures around 12℃ [1]. The isolation of *Ferrimicrobium* species with a pH optimum around pH 2 and a temperature optimum around 35℃ as described before [2] may be explained by the occurrence of hot spots due to pyrite oxidation [19].

Due to the amplification of archaeal 16S rRNA genes from the samples in 2006 and 2008and sequencing with 99 percent identity for uncultured and unidentified archaea deposited in NCBI databases, the growth experiments were performed at increased temperatures up to 70℃. At 45℃ from sand samples in 2006 no growth was obvious in media containing 10 g/L ferrous iron or elemental sulphur. Also the addition of up to 0.5 percent yeast extract to the media showed no improvement of this cultivation procedure. From the samples taken 2008 growth for iron-oxidizing microorganisms was visible in two cases, but cultivation for sulphur-oxidizing microorganisms failed so far. By the double agar plate technique pure cultures of iron-oxidizing bacteria were achieved and identified by sequencing of the 16S rRNA genes as *Sulfobacillus acidophilus* with 99 percent identity.

The detected growth of iron-oxidizing microorganisms in the sand samples may be explained by increased temperatures probably by pyrite oxidation as described before [19]. This is supported by the isolation of *S. acidophilus*, described with a temperature optimum around 45 to 50℃ [20].

As described before, at 70℃ growth from one sample from 2006 in media with DSMZ designation 182 was obvious, but sustained cultivation longer than six months or isolation of pure cultures failed [1]. Microscopic observation revealed growth of a bacterial mixed culture, which was supported by the sequencing of the amplified bacterial 16S rRNA genes after DNA extraction, whereas amplification of archaeal 16S rRNA genes failed.

From 4 of the 42 samples from 2006 and 2008 different pure cultures were achieved and identified as *Stenotrophomonas maltophilia*, *Acinetobacter calcoaceticus*, and *Geobacillus caldoxylosilyticus* by sequencing of the 16S rRNA genes. The three species are known soil bacteria, but not involved in bioleaching [21, 22].

The isolation from 70℃ cultures is surprising, but explainable on the example of the pathogen *Stenotrophomonas maltophilia* with the resistance to heavy metals and antibiotic treatment [23, 24].

Two years after the first analysis of a Lusatian mining area, leaching bacteria were detected by cultivation and molecular biology based methods. Up to now the search for new microorganisms is in progress with the identification of the iron-oxidizing microorganisms which grow at 45℃, as well as the characterisation of the unidentified archaeal 16S rRNA gene amplificates.

4 Conclusion

Two years after the first sampling in a lignite mining area in Lusatia *A. ferrooxidans* and *A. thiooxidans* were detected again. First isolation of *A. albertensis* and *Ferrimicrobium* sp. broaden the spectrum of leaching bacteria there. The first isolation of *Sulfobacillus acidophilus* at 45℃ encourages the search for new microorganisms since two mixed cultures grew. The knowledge of the species will be a basis to develop a strategy to inhibit AMD.

Table 2 Cultivation results at 28℃ or 45℃ for the identification of iron- and sulphur- oxidizing microorganisms from sampling campaigns in 2006 and 2008 (51°80′N; 14°50′E). Cultivation of sulphur-oxidizing (SoB) and iron-oxidizing (FeoB) microorganisms occurred in media containing 10 g/L ferrous iron, or elementary sulphur [9, 10]. Purification was performed with double-agar plates as described [12] and identification was performed by sequencing of the 16S rRNA gene amplificates. Listed are the ratios of positive samples/total samples, and the identified species.

Year	Mixed cultures/total samples			Species identified in pure cultures by sequencing 16S rRNA gene amplificates
	Temp.	SoB	FeoB	
2006	28℃	27/32	24/32	*A. ferrooxidans*, *A. thiooxidans*, *L. ferrooxidans*, *L. ferriphilium*, *F. acidiphilium* and *A. acidophilium*
2008		10/10	10/10	*A. ferrooxidans*, *A. thiooxidans*, *A. albertensis*, *Ferrimicrobium* sp.
2006	45℃	0/32	0/32	no pure cultures achieved
2008		0/10	2/10	*S. acidophilus* / in progress

Acknowledgment

We are grateful to Mitteldeutsche Braunkohlengesellschaft mbH, Vattenfall Europe Mining AG and GMB GmbH for financial support.

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4.2.3 Effect of SDS on planctonic *Acidithiobacillus thiooxidans* and bioleaching of sand samples

Siebert, H.M., R. Marmulla, and K.P. Stahmann (2011)
Minerals Engineering, 24(11):1128-1131

Minerals Engineering 24 (2011) 1128–1131

Contents lists available at ScienceDirect

Minerals Engineering

journal homepage: www.elsevier.com/locate/mineng

Effect of SDS on planctonic *Acidithiobacillus thiooxidans* and bioleaching of sand samples

Hans-Michael Siebert, Robert Marmulla, K.-Peter Stahmann *

University of Applied Science Lausitz, Hochschule Lausitz (FH), Bio-, Chemie- und Verfahrenstechnik, Großenhainer Str. 57, D-01968 Senftenberg, Germany

ARTICLE INFO

Article history:
Available online 5 April 2011

Keywords:
Acid rock drainage
Bioleaching
Bacteria
Environmental

ABSTRACT

The inhibition of bioleaching by sodium dodecyl sulphate (SDS), previously used in large scale percolators in Romania (Schippers et al., 2001), was shown for pure cultures of sulphur-oxidizing *Acidithiobacillus thiooxidans* DSM 622 or German sand samples.

A decrease of 25–75% in planctonic cell number from an initial 10^{10} *A. thiooxidans* cells, counted with a Thoma-chamber 30 min after exposure to SDS concentrations from 0.5 to 10 g/L, suggested a cell lysis. Additionally a release of nucleic acids was found.

To apply these results in a more complex habitat, columns filled with aquifer material from an East German lignite mining area containing 1% pyrite were treated. Columns were washed once with 2 g/L SDS and afterwards with rainwater. Most-probable-number determinations of flow-through revealed no growth of iron- and sulphur-oxidizing microorganisms within 26 weeks, while up to 10^6 cells per millilitre were determined in the control. Elution of sulphate dropped to 25%.

1. Introduction

The oxidation of sulphide containing minerals, e.g. pyrite and marcasite, leads to sulphuric acid containing products. The dissolved metals contaminate ground and surface water, a phenomenon known as acid mine drainage (AMD). Microorganisms mediate and accelerate this process. Occurrence of microorganisms and mechanisms of bacterial sulphide oxidation in AMD areas were reviewed by Rohwerder et al. (2003). In a study of an East German lignite mining area, carried out in 2006, the acidophilic iron- and sulphur-oxidizing bacteria *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans* were identified. In overburden, *A. ferrooxidans* and *A. thiooxidans* were equally distributed, in heap samples *A. ferrooxidans* was the dominant acidophile (Siebert et al., 2009).

Inhibition of bacterial and chemical leaching is necessary to protect the environment from AMD. The application of sulphonated tensides like sodium dodecyl sulphate (SDS), resulting in inhibition or enhancement, was previously described (Onysoko et al., 1984; Sand, 1985; Dugan, 1986). Schippers et al. (2001) showed the efficient inhibition of leaching bacteria by SDS application and introduction of alkaline layers in large percolators. Effects of other substances, e.g. quaternary ammonium compounds or hydrocarbons, to planctonic microorganisms were described by Sikkema et al. (1995), Kourai et al. (2006), and Sumitomo et al. (2006). The influence of surface active compounds on the adhesion of microorganisms to surfaces was reviewed by Neu (1996).

Cell lysis in Gram-positive and -negative bacteria and various ways of its induction have been studied for more than 100 years (reviewed by Rice and Bayles, 2008). Tsuchido et al. (1985) showed that lysis in *Bacillus subtilis* was triggered by long chain fatty acids. An induced release of genomic DNA as well as the development of competence in bacterial populations in the lytic stage were also reported (Steinmoen et al., 2002; Kreth et al., 2009).

The objective of this study was a first approach to collect evidence for a lysis mechanism explaining the sensitivity of bioleaching for an inhibition by SDS.

2. Material and methods

2.1. SDS treatment of high density cell suspension of A. thiooxidans

2.1.1. Microorganism cultivation

The sulphur-oxidizing bacterium *A. thiooxidans* DSM 622 was pre-cultivated in a medium according to Hutchinson et al. (1965), containing 10 g/L sulphur. For lysis experiments cultures were transferred into a modified medium according to Mackintosh (1978), containing 1 mM ammonium chloride instead of ammonium sulphate, and 5 g/L sulphur.

2.1.2. Experimental design and analysis

Cells were cultured until the late logarithmic phase and harvested by centrifugation. Afterwards, a bacterial suspension was

* Corresponding author. Tel.: +49 3573 85 801/913; fax: +49 3573 85 809.
E-mail address: Klaus-Peter.Stahmann@hs-lausitz.de (K.-P. Stahmann).

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doi:10.1016/j.mineng.2011.03.003

adjusted to 10^{10} cells per millilitre with fresh medium. As inhibitory substance, SDS was dissolved in double distilled water to a 10% stock solution, diluted to appropriate concentrations and filter-sterilized. Equal volumes of SDS dilutions were added to 1 mL of suspension for final concentrations of 0.5, 1, 2.5, 5, or 10 g/L. Solutions were mixed and incubated for 30 min at room temperature. Cell number determination was performed in a Thoma-chamber with appropriate dilutions, before and after SDS treatment. All reported data are mean values of duplicate measurements. The percentages given as results were calculated in reference to the appropriate negative control.

For analysis of nucleic acid release, 500 μL of bacterial suspensions after SDS treatment were centrifuged at 13,000*g* for 10 min. Nucleic acids in the supernatants were precipitated by isopropanol, washed with 70% ethanol and suspended in 20 μL double distilled water. Cell pellets were suspended in 20 μL double distilled water. Equal amounts of pellet or supernatant samples were analysed by electrophoresis on 0.8% agarose gels and stained with ethidium bromide.

2.2. Column experiments

For leaching experiments, 20 mL columns (Bio-Rad, USA) were used. Up to 16 g aquifer material, containing 0.4–1% pyrite, from an East German lignite mining area were filled into the columns. The experiments were performed at 21 °C.

For inhibition experiments, SDS was solubilised in rainwater to a final concentration of 2 g/L. The columns were washed in two steps. Firstly, with 85 mL SDS-rainwater mixture or just rainwater for the control. That was carried out in eight charges over a period of seven days. To get best possible contact with the material each charge was applied resulting in a liquid phase above the sand sample. Afterwards, the columns were washed with 5 mL rainwater applied in one charge per week. The eluent was collected for 1 week, adjusted to 5 mL with fresh rainwater, and again applied to the column. As a positive control, a further column was loaded every week with 5 mL fresh rainwater.

Sulphate measurement of the leaching solution was performed once per month with Spectroquant® test (No. 1.14791.0001; Merck, Germany), following manufacturer's instructions. Additionally, pH value was measured every 2–4 weeks. Measurement of ferrous and total iron content from the leaching solution was performed after 23 weeks with Spectroquant® test (No. 1.00796.0001; Merck, Germany), following manufacturer's instructions. The content of ferric iron was calculated as difference of total and ferrous iron.

Cell counts of acidophilic iron- and sulphur-oxidizing microorganisms were determined by most-probable-number technique in liquid media according to Mackintosh (1978) and Hutchinson et al. (1965). Cultures were incubated at 28 °C for 3–4 weeks. Sand samples were analysed before the experiment and 26 weeks after SDS addition to the column. The leaching solution was additionally sampled after 13 weeks.

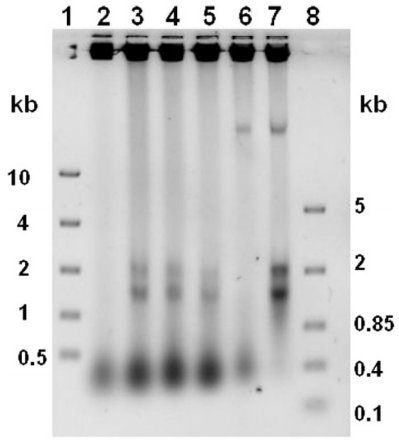

Fig. 2. Agarose gel of nucleic acids released from *A. thiooxidans* pellet fraction after SDS treatment. Lanes: (1) high range marker (Fermentas); (2) control without SDS; (3) 0.5 g/L SDS; (4) 1 g/L SDS; (5) 2.5 g/L SDS; (6) 5 g/L SDS; (7) 10 g/L; (8) middle range marker (Fermentas).

3. Results and discussion

3.1. SDS caused cell number decrease and nucleic acid release

Planctonic cells of *A. thiooxidans* DSM 622, counted 30 min after exposure to 0.5–10 g/L SDS, showed a decrease in cell number between 25% and 75% compared to the initial cell count (Fig. 1). High molecular weight nucleic acids above 10 kb were released in the pellet fraction after addition of 5 or 10 g/L SDS. But, most of the nucleic acids were stacking in the slots adhering to the cell debris. Lower SDS concentrations resulted only in a release of nucleic acids up to 2 kb (Fig. 2). Additionally, in comparison to the control sample more nucleic acids below 500 bp were visible. In the supernatant nucleic acids were not detectable (data not shown). Since the addition of SDS does not only permeabilise the cytoplasma membrane, but also influences the adsorption forces between the macromolecules, which was obvious by a high viscosity of the suspended pellets applied to the gel slots, there are probably overlapping effects. This might explain that, firstly, the nucleic acids running at 2 kb were decreasing from 0.5 to 5 g/L and at high concentrations appearing again. Secondly, the low molecular weight

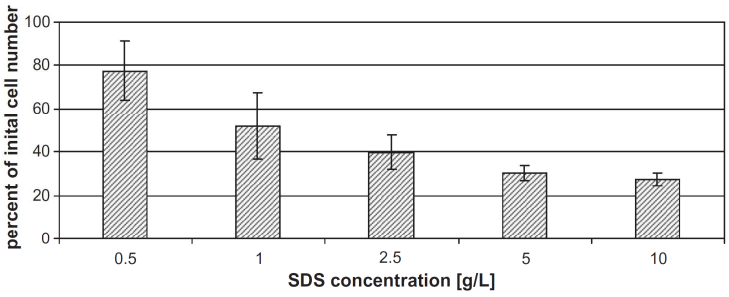

Fig. 1. Lysis of planctonic *A. thiooxidans* DSM 622. Cell number counted 30 min after exposition to different concentrations of SDS in relation to the control.

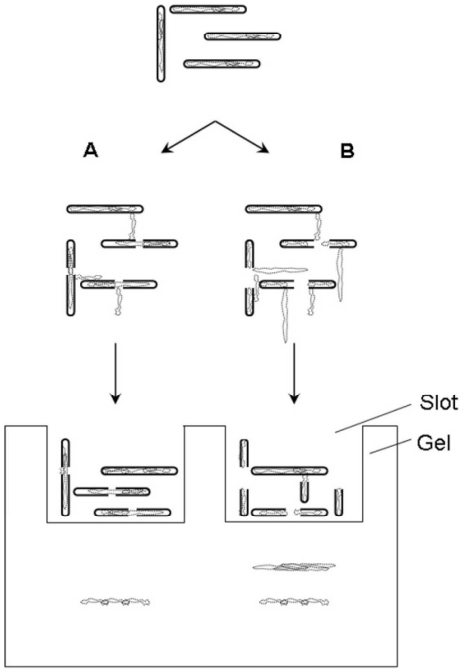

Fig. 3. Hypothesis for SDS triggered release of nucleic acids from *A. thiooxidans* rods and separation in an agarose gel. Scheme on top shows *A. thiooxidans* culture after centrifugation, scheme A in the middle part shows SDS triggered release of low molecular weight nucleic acids, attached to cells, after treatment with SDS concentrations from 0.5 to 2 g/L. Scheme B in the middle shows SDS triggered release of high, as well as low molecular weight nucleic acids, attached to cells, after treatment with 5 or 10 g/L SDS. Scheme on the bottom shows the separation of nucleic acids from bacterial cells by electrophoresis through an agarose gel.

nucleic acid running at 0.4 kb was disappearing. Since it was not found in the supernatant, it must remain with or within the cell debries in the slots. Decrease in cell number and survival of a subpopulation of bacteria after autolysis was described by Qin et al. (2007) for *Staphylococcus epidermis*. The *Staphylococcus* wild-type showed a 30% decrease of the initial OD_{580} after treatment with Triton X-100, which is similar to our observation at 0.5 g/L SDS.

It has been shown that release of nucleic acids is important in biofilm formation and cell–cell adhesion (Allesen-Holm et al., 2006; Qin et al., 2007; Rice et al., 2007). Since *A. thiooxidans* is known as a biofilm producer, this microorganism should possess a mechanism for secretion of nucleic acids into the biofilm. The release of low as well as high molecular weight nucleic acids triggered by SDS, shown for *A. thiooxidans* in this study for the first time, fits to that biofilm model (Fig. 3). Release of nucleic acids by external influence was described by Kreth et al. (2009), showing that hydrogen peroxide induced DNA release in *Streptococcus sanguinis* and *Streptococcus gordonii*. The induction of a competent state initiated release of DNA from a *Streptococcus pneumoniae* subpopulation was shown by Steinmoen et al. (2002). This DNA can be taken up by competent cells. This would explain the 25% not-lysed subpopulation of *A. thiooxidans* even after treatment with 10 g/L SDS.

3.2. SDS treatment inhibited bioleaching

To apply these results in a habitat that is more complex due to microbial diversity, biofilms, and solid substrates, experiments were performed in 20 mL columns. In laboratory scale these volumes can be easily handled. The columns were filled with material containing iron- and sulphur-oxidizing microorganisms in concentrations between 1×10^4 and 5×10^4 cells per gram sand. After 26 weeks in control columns with rainwater, around two orders of magnitude more iron-oxidizing microorganisms were measured per gram sand. Sulphur-oxidizing microorganisms were found in the range from 1×10^2 to 1×10^5 cells per gram sand, estimated by most-probable-number technique, for fresh and reused rainwater. In the SDS treated sample, iron- and sulphur-oxidizing microorganisms were not detectable. In leaching solution of the control columns 13 weeks after start of the experiments, iron- and sulphur-oxidizing microorganisms were found in concentrations between 1×10^4 and 5×10^5 cells per millilitre (Fig. 4). After an additional 13 weeks, both controls showed a slight decrease of iron-oxidizing microorganisms. Sulphur-oxidizing microbes were found in concentrations of 1×10^5 or 5×10^1 cells per millilitre for fresh or reused rainwater, respectively. In the leaching solution of the SDS treated sample iron- and sulphur-oxidizing microorganisms were not detectable over the complete run. The decrease below the detection limit of leaching microorganisms after SDS treatment correlates with studies in column experiments with addition of 1 mM SDS (Schippers et al., 1998). Repeated SDS treatment performed by Schippers et al. (2001) resulted in long term inhibition of leaching microorganisms in 65 m^3 percolators. The rapid decrease of leaching microorganisms after a single SDS treatment in our experiments is explainable by higher surfactant concentration and the scale of our columns, which are six orders of magnitudes smaller, allowing faster contact.

The control washed with fresh rainwater showed pH values around 4.0 for 4 weeks, then dropped to pH 2.0. In the sample with reused rainwater, pH values dropped within 12 weeks from 3.0 to 1.5. The SDS treated sample showed over the entire run pH values

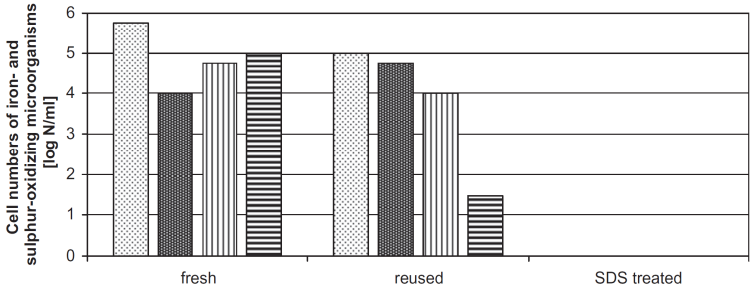

Fig. 4. Cell number determined by most-probable-number technique from leaching solution of three columns. Cultivation at 28 °C for 3–4 weeks in medium according to Mackintosh (1978) for iron-oxidizing microorganisms (▨ 13 weeks, ■ 26 weeks) or Hutchinson et al. (1965) for sulphur-oxidizing microorganisms (▥ 13 weeks, ▤ 26 weeks).

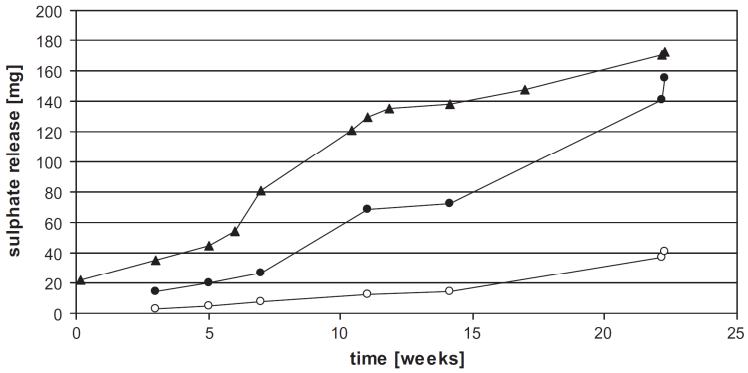

Fig. 5. Time course of sulphate released from 20 mL sand columns run with fresh (—▲—), reused (●) or 2 g/L SDS containing (○) rainwater.

Table 1
Determination of ferrous and total iron content from leaching solution of the three 20 mL sand columns after 23 weeks. Ferric iron content was calculated as difference of total and ferrous iron.

	Concentration (mg/L)			Ratio (%)
Samples	Fe(II)	Fe_{total}	$Fe(III)_{calc.}$	$Fe(III)/Fe_{total}$
Fresh	26	1.260	1.234	2.1
Reused	34	3.018	2.984	1.1
SDS treated	167	179	12	93.4

between 4.5 and 4.0. In the SDS treated column a decrease of sulphate release of more than 75% was measured, compared to the controls (Fig. 5). In the leaching solution of the SDS treated column the ratio of ferric to total iron was calculated to 93%, compared to 1–2% of the controls (Table 1). This supports the results shown above. A stable pH around 4 and a reduction in sulphate release after SDS treatment over 26 weeks indicate long term inhibition of leaching microorganisms in column scale experiments.

4. Conclusions

Release of DNA and significant but not complete disappearance of *A. thiooxidans* cells after SDS treatment suggest a lysis mechanism for the planctonic high cell density system. In the more complex sand samples neither sulphur- nor iron-oxidizing microorganisms were detectable and sulphate release reduced by SDS treatment indicating the sensitivity of bioleaching.

Acknowledgements

We are grateful to Wolfgang Sand for providing the *A. thiooxidans* strain and helpful discussions. Furthermore we would like to thank Mitteldeutsche Braunkohlengesellschaft mbH, Vattenfall Europe Mining AG and GMB GmbH for the provision of sand samples and for financial support.

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4.3 Eingereichte Manuskripte

4.3.1 Iron- and sulphur- oxidizing microorganisms in drilling cores, overburden and heaps of five lignite mining areas in Germany

Siebert, H.-M., B. Florian, J. Havenstein, S. Krüger, W. Sand, and K.-P. Stahmann

Eingereicht bei Applied and Environmental Microbiology

Iron- and sulphur- oxidizing microorganisms in drilling cores, overburden and heaps of five lignite mining areas in Germany

Hans-Michael Siebert[1*], Bianca Florian[2], Julia Havenstein[1], Susann Krüger[1], Wolfgang Sand[2], K.-Peter Stahmann[1]

[1] Fakultät für Naturwissenschaften, Hochschule Lausitz (FH), Großenhainer Str. 57, D-01968 Senftenberg, Germany

[2] Aquatische Biotechnologie, Biofilm Centre, Fakultät für Chemie, Universität Duisburg-Essen, Universitätsstraße 2, D-45141 Essen, Germany

Keywords: Biodiversity, 16S rRNA gene, *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Leptospirillum* spp.

Running title: **Leaching bacteria in five german lignite mining areas**

* Corresponding author, Postal adress: Fakultät für Naturwissenschaften, Hochschule Lausitz (FH), Großenhainer Str. 57, 01958 Senftenberg, Germany. Phone: +49-3573-85-935 / 801. Fax: +49-3573-85-809.

Mailing address: Hans-Michael.Siebert@hs-lausitz.de

Abstract

Acid mine drainage with sulphate release and low pH due to the oxidation of sulphide minerals is accompanied with lignite mining. This triggered a study in five mining areas in Lusatia and central Germany with sampling campaigns from 2006 to 2009. From the freshly liberated overburden exposed to the air for one hour and from the sand layer below the lignite, as well as at optically striking sites, 66 samples were taken. DNA encoding 16S rRNA of *Acidithiobacillus ferrooxidans* was detected in 46 cases, whereas that of *A. thiooxidans* was found in 36 samples. *Leptospirillum* spp. DNA was detected only in 14 cases. Cultivation of iron- or sulphur- oxidizing microorganisms was successful from 53 or 57 samples, respectively. Pure cultures of mesophilic and moderate thermophilic bacteria were achieved in 168 cases. Most were identified by 16S rRNA genes as the above mentioned species. Additionally, *L. ferrooxidans*, *L. ferriphilum*, *Acidiphilium acidophilum*, *Ferrimicrobium acidiphilum*, *Sulfobacillus acidophilus*, *S. thermosulfidooxidans*, and *S. thermotholerans*. were found.

To clarify the starting point of bioleaching, samples were taken from drilling cores, either before, or after groundwater lowering. The leaching bacteria *A. ferrooxidans*, *A. thiooxidans*, and *Leptospirillum* spp. were detected by cultivation or by amplification and sequencing of the 16S rRNA genes in the samples after lowering the groundwater level. In samples which were taken before the groundwater lowering only by cultivation in 1/24 or 8/24 samples iron- or sulphur- oxidizing microorganisms were detected, respectively. These findings support the idea that the bioleaching starts by lowering the groundwater.

48 **Introduction**

49 In Germany there are three lignite mining areas in the eastern, central and western part of the
50 country. The mining areas in Lusatia and central Germany are located in Brandenburg,
51 Saxony and Saxony- Anhalt and the lignite has been excavated since the 17th century. In
52 these mining areas flooded open cast mines exist and were characterized by a low pH value
53 and high sulphate concentrations (13), caused by acid mine drainage (AMD). In this
54 biologically mediated process sulphide minerals e.g. pyrite and marcasite in the overburden
55 were oxidized (10). This reaction leads to sulphuric acid, containing dissolved heavy metal
56 ions, contaminating the surface and ground water. The occurrence of the iron- and sulphur-
57 oxidizing microorganisms were described to mediate and accelerate these processes (3, 35).
58 Examples for these leaching microorganisms are the well studied leaching bacteria
59 *Acidithiobacillus ferrooxidans* and *A. thiooxidans*, as well as the iron- oxidizing bacteria from
60 genus *Leptospirillum* spp. and the heterotrophic *Acidiphilium* spp., as reviewed by Rohwerder
61 *et al.* (26). Furthermore, archaea were found in mining associated areas and are known to live
62 in extreme environments characterized by e.g. low pH value and temperatures above 65°C.
63 Examples for the thermophilic archaea are from the genus *Acidianus* spp., *Sulfolobus* spp.
64 (11, 12), but also mesophilic archaea from the order *Thermoplasmales* were described (9).
65
66 The most frequently used antimicrobials to inhibit the leaching bacteria and consequently the
67 AMD are biocides e.g. thiazolinone derivates, quaternary ammonium salts and detergents like
68 sodium dodecyl sulphate (SDS). The treatment of planktonic cells with SDS was described
69 before and resulted in inhibition (7, 24) or enhancement of the bacterial leaching (27). Other
70 examinations showed an efficient inhibition of leaching bacteria with substances e.g.
71 quaternary ammonium compounds or hydrocarbons (19, 33). The long term inhibition of
72 leaching bacteria in large percolators by SDS treatment and by neutralizing agent was also
73 described (28, 29). Another chemical method for the AMD treatment is the addition of

alkaline chemicals like limestone ($CaCO_3$) or soda ash (Na_2CO_3) (36) resulting in a neutralization of the pH.

Aim of this study was the detection of iron- and sulphur oxidizing microorganisms in five lignite mining areas in Lusatia and in central Germany. For the detection of the microorganisms three independent methods were used. Furthermore, elementary analyses for heavy metals or iron, sulphide and sulphate were performed. To understand where the start of the bioleaching is, sampling campaigns for drilling cores before and after lowering the ground water level were performed. These samples were also examined by classical cultivation based technique for the isolation and characterisation of the microorganisms. Furthermore, molecular biology based techniques were used and the metabolic activity was measured by microcalorimetry.

Material and Methods

Sampling

In Germany mining areas exist in the Rhineland, Lusatia and in the central part of Germany. In this study different sampling campaigns were performed from 2006 to 2008 from three Lusatian mining areas and two mining areas in central Germany (Fig. 1). In the first campaign in 2006 in area B 1, samples were taken from section or from heap area for an age profile with an exposition to atmospheric oxygen for up to 30 years after removing the overburden by the overburden conveyor bridge F60. Additionally, samples were taken from overburden at different level of the tertiary strata above the lignite. Furthermore, samples were taken at optically striking sites e.g. with yellow sand incrustations or oily brown efflux (Fig. 2). For an enlargement of the database further sampling campaigns were preformed in four mining areas (Tab. 1). Therefore in the Lusatian areas (B 1, B 2, S 1) samples were taken from overburden up to one hour after removing of the sand layer by the overburden conveyor bridge F60, two

100 to ten meters above the lignite. In mining areas in central Germany (S 2, A 1) material were
101 taken from the tertiary strata from sand, clay or coarse clay. Furthermore, in the mining areas
102 samples were taken at optically striking sites with e.g. a brown efflux. For sampling the upper
103 30 to 50 cm dry sand layer was removed, than 250 g sample was taken (Fig. 2). The mapping
104 of the samples was performed by global positioning system measurement.
105
106 Additionally to the sampling of active mining areas in Germany, sampling of drilling cores
107 before and after lowering of ground water level was performed (Fig. 1). The drilling cores DC
108 1 and DC 2 were taken from an area with unaffected ground water level. Drilling cores DC 5
109 and DC 6 were located near mining area S 2 and therefore longer exposed to lowering of the
110 ground water level, than DC 3 and DC 4 (Tab. 2). For sampling around 250 g material was
111 taken during the drilling process (Fig. 3). The samples were exposed to the air between one
112 hour and five days.
113
114 **Chemical analysis**
115 From area B 1 the samples taken in 2006 were analyzed for sulphide, sulphate and iron by the
116 Potsdamer Wasser und Umwelt Labor GmbH & Co. KG (Potsdam, Germany). The pyrite
117 content was calculated from these values. Additionally from two sand samples, from one day
118 old section or 30 years old heap, an analysis of sulphur, iron and trace elements was
119 performed by the Institut für komplexe Werkstoffe (Dresden, Germany).
120
121 **Detection of the metabolic activity**
122 For the microcalorimetric measurements calorimeters Thermometric 2277 Thermal Activity
123 monitor (New Castle, United States) and LKB Bromma 2277 BioActivity monitor (LKB,
124 Sweden) were used, heated with a Thermometric 5510 thermostat or an LKB 2219 multi-temp
125 II thermostatic circulator.

126 The experiments were accomplished as two- step measurement. The first measurement with
127 the untreated sand sample was carried out to determine the entire activity i.e. biological and
128 chemical activity. After heat inactivation of mesophilic microorganisms at 60°C the
129 remaining activity of the sand sample was determined by microcalorimetry. The difference of
130 the two measurements resulted in the energy delivered by the microbial metabolism, carried
131 out as described before (30).
132
133 **Molecular biological detection of microorganisms**
134 From all samples the molecular biological experiments were performed after extraction of the
135 nucleic acids directly from the material. Around 7.5 g sample was suspended in 13 mL DNA-
136 extraction buffer (100 mM Tris/HCl, 100 mM EDTA, 100 mM NaH_2PO_4, 1.5 M NaCl, 1%
137 (w/v) CTAB, pH 8), as described previously (41). Microorganisms in the samples were lysed
138 by four cycles freeze thawing. Afterwards 100 µL of proteinase K solution (20 mg/mL) was
139 added and the samples were incubated for 30 minutes at 37°C. Subsequently, 1.5 mL 20%
140 (w/v) SDS solution was added. After two hours incubation at 65°C, samples were centrifuged
141 at 15,000 x *g* for ten minutes. Supernatant was stored, the sediment was suspended again in
142 12.5 mL DNA- extraction buffer added with 1.25 mL 20% (w/v) SDS solution and incubated
143 for ten minutes at 65°C. Afterwards the samples were centrifuged again at up to 15,000 x *g*
144 for ten minutes. The supernatants were pooled and extracted twice with one volume
145 chloroform: isoamylalcohol (25:1). Subsequently, nucleic acids were precipitated with one
146 volume isopropanol and centrifugation for 30 minutes at 15,000 x *g*. The pellet was washed
147 twice in 70% ethanol, dried and finally suspended in 300 µL TE- buffer (10 mM Tris/HCl, 0.1
148 mM EDTA, pH 8). The concentrations of the obtained genomic material were visualized by
149 staining with ethidium bromide after electrophoresis on 0.8% (w/v) agarose gel (Fig. 4).
150 Extracted DNA was further purified from humic substances and PCR interfering substances

by PowerClean DNA Clean-up Kit (No. 12877-50, MoBio Laboratories Inc., United States). Purified DNA was diluted 1:10 or 1:100 and used as template for PCR experiments.

Genomic material from planktonic cultures was extracted as described before (21). Up to two millilitres of bacterial culture was centrifuged for 20 minutes at 13,000 x *g* and washed twice with phosphate buffered saline. Afterwards microorganisms were suspended in 500 µL buffer A (100 mM NaCl, 100 mM Tris, 1 mM sodium citrate, 5 mM $CaCl_2$, 25 mM EDTA, pH 8.0). Four cycles freeze thawing were performed, if extraction of nucleic acids from microorganisms was not successful. Subsequently, 20 µL polyadenylic acid potassium salt (10 mg/mL), 20 µL 10% (w/v) tetra- sodium diphosphate decahydrate and 30 µL lysozyme (100 mg/mL) were added and incubated for 40 minutes at 37°C. Followed by the addition of 60 µL proteinase K (100 mg/mL) and 10 µL 20% (w/v) SDS, incubation for 30 minutes at 50°C was performed. Nucleic acids were extracted by addition of 200 µL 20% (w/v) SDS and 600 µL chloroform: isoamylalcohol (25:1) and precipitated with isopropanol. The pellets were washed with 70% ethanol, dried and dissolved in 30 to 50 µL water or TE-buffer.

For analysis of the bacterial or archaeal DNA 16S rRNA gene amplification was performed with different protocols and primer pairs (Tab. 3 and Fig. 5). The experiments were performed in 50 µL reaction mixture consisting of 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP Mix, 0.5 µM of each primer, one unit Taq DNA polymerase (Jena Bioscience, Germany) and purified DNA. For the amplification of the archaeal 16S rRNA genes 2 mM $MgCl_2$ and additional 5% dimethyl sulphoxide (DMSO) were added to the reaction mixture.

Detection of the bacterial 16S rRNA genes was performed with primer pairs 8For and 1492Rev and a protocol was used as described before (23). The amplification of the archaeal 16S rRNA gene was performed with primer pair Arch21F and Arch958R and a protocol as

177 described before (4). For the detection of leaching bacteria on genus or species level two
178 different PCR protocols were used (5, 21). For the amplification of *Leptospirillum* spp.
179 specific amplificates primer pair LFer-P1 and NR-R2 was used, whereas *A. ferrooxidans* and
180 *A. thiooxidans* specific amplificates were achieved with forward primers Ferro458F or
181 Thio458R, respectively and Ferro1473R as reverse primer. For the amplification of the
182 heterotrophic *Acidiphilium* spp. the primer pair Acido594F and Acido958R was used. For
183 verification of the 16S rRNA gene amplificates sequencing was performed from all pure
184 cultures and amplificates achieved directly from the sand samples were randomly sequenced.
185
186 **Cultivation experiments**
187 Samples were examined for mesophilic to thermophilic, autotrophic or heterotrophic iron- and
188 sulphur- oxidizing microorganisms and cultivation experiments were performed at 28, 45, or
189 70°C, respectively. The cultivation for mesophilic iron- and sulphur- oxidizing
190 microorganisms was performed in test tubes containing 10 g ferrous sulphate or 10 g
191 elementary sulphur per litre, in media as described (22, 15). Additionally, for cultivation of
192 the thermophiles the media were supplemented with 0.1 to 0.5% yeast extract, or with 9K-
193 medium and 10 g/L ferrous sulphate, supplemented with or without 2.5 mM potassium
194 tetrathionate (34). The isolation of acidophilic microorganisms was performed by repeated
195 dilution series in 96- well plates. A further method used for the isolation of mesophilic pure
196 cultures was the repeated isolation of single colonies by the double- agar- plate technique as
197 described before (16). Isolation of microorganisms at temperatures above 45°C was
198 performed in Petri dishes by addition of up to 0.8% gellan gum to liquid media.
199 For long term storage of the isolated pure cultures, the microorganisms were centrifuged for
200 20 minutes at 22,000 x *g*, suspended in basal salt medium without ferrous iron (22), with the
201 addition of 7% DMSO, and immediately frozen in liquid nitrogen and stored at -80°C (14).
202

203 **Results**

204 **Pyrite oxidation in a Lusatian mining area**

205 A first inventory of microorganisms in a Lusatian open lignite mining area B 1, was
206 performed in 2006 and a second in 2008 (31, 32). In both sampling campaigns the leaching
207 microorganisms were detected in almost all systematic as well as in the optically striking
208 samples by molecular and cultivation based techniques (Tab. 4). Detection of these
209 microorganisms corresponds to the heat output measured by microcalorimetry, with up to
210 100% microbial activity. In the samples from 2008 microcalorimetric activity up to 62 µW
211 was found in the systematic samples. From the samples at optically striking sites a more than
212 four times higher microcalorimetric activity was measured.
213 From sampling in 2006 chemical analysis for iron, sulphate and sulphide was performed from
214 all samples. In freshly liberated samples exposed to the air for one day relatively low sulphate
215 and high iron concentrations were obvious (Tab. 5). With increased exposition to atmospheric
216 oxygen for up to 30 years in the overburden increased sulphate content was found with a
217 maximum of more than 11 g/kg and the sulphide content was below the detection level of 1.5
218 mg/kg sand. Also decreased iron content with 9.62 g/kg sand was obvious. In the old heap
219 samples, where the overburden was mixed from layers of different depths, the differences in
220 sulphate, sulphide, and iron content increased with the age of the samples. These findings
221 were supported by elementary analysis of two samples from the fresh liberated overburden,
222 exposed to the air for one day or from a 30 years old heap sample (Tab. 6). In the fresh
223 sample the content of iron and heavy metals, as well as sulphur was higher compared to the
224 older heap sample.
225

226 **Occurrence of iron- and sulphur- oxidizing microorganisms in active lignite mining**
227 **areas in Lusatia and central Germany**

228 For an enlargement of the database and supporting of the sampling campaigns in 2006 and
229 2008 in the mining area B 1, additionally 24 samples were taken in the four other mining
230 areas and examined for leaching microorganisms by three independent methods (Tab. 7). For
231 the molecular based detection of leaching microorganisms extraction of nucleic acids direct
232 from the samples was necessary and successful from 17 of the 24 samples, visible by staining
233 with ethidium bromide after electrophoresis through an agarose gel. After purification from
234 PCR interfering substances from 16 of the samples a specific amplificate for bacterial 16S
235 rRNA gene was found. The leaching bacteria *A. ferrooxidans* and *A. thiooxidans* were equally
236 distributed in the samples from the four mining areas, with the detection in 14 or 18 cases,
237 respectively. Differences between the systematic or optically striking samples were not
238 obvious (Tab. 7). Specific amplificates for *Leptospirillum* spp. were found in eight of the
239 systematic and none of the striking samples. The molecular based detection of iron- and
240 sulphur- oxidizing bacteria was supported by cultivation experiments at 28 and 45°C. In the
241 samples with the molecular based detection of *A. ferrooxidans* and *A. thiooxidans* growth at
242 28°C was obvious for iron- and sulphur- oxidizing microorganisms. Cultivation experiments
243 at elevated temperatures of 45 to 70°C showed in two samples at 45°C signals for iron- and in
244 one sample for sulphur- oxidizing microorganisms, respectively. Additionally, 168 pure
245 cultures, cultivated at 28 and 45°C, were achieved by dilution series and by isolation of single
246 colonies by the double- agar- plate technique. The cultures were identified by amplification
247 and sequencing of the 16S rRNA genes as *A. ferrooxidans*, *A. thiooxidans*, *L. ferrooxidans*, *L.*
248 *ferriphilum*, *Ferrimicrobium* sp., *Sulfobacillus acidophilus*, *S. thermotolerans*, and *S.*
249 *thermosulfidooxidans*. The occurrence of the leaching bacteria was also supported by
250 metabolic activity between 24 and 299, or maximal 33 µW per gram material in the samples
251 from Lusatian or mining areas in central Germany, respectively.
252

253 **Occurrence of leaching bacteria in drilling cores before or after lowering of the ground**
254 **water level**

255 For the clarification of the first occurrence of leaching microorganisms and the start of the
256 microbial pyrite oxidation, drilling cores before and after lowering of the ground water level
257 were examined.

258 Cultivation for iron- and sulphur- oxidizing microorganisms in the 42 samples from drilling
259 cores after lowering of the ground water level revealed growth in two or 12 cases,
260 respectively (Tab. 8). The main occurrence of the sulphur- oxidizing microorganisms was
261 detected in six cases in the sand and in the lignite layer with three cases, respectively.
262 Interesting hereby was the detection ratio of iron- and sulphur- oxidizing microorganisms
263 with three to 11 between the drilling cores DC 3 and DC 4 and drilling cores DC 5 and DC 6
264 which had a longer time of ground water lowering. The metabolic activity was measured in
265 DC 3 and DC 4 with 125 and 319 µW per gram material, whereas in DC 5 and DC 6
266 biological with 90 or 29 µW per gram material was measured. Lower detection rates were
267 found from drilling core samples before lowering the ground water level with only one signal
268 for iron- oxidizing microorganisms (Tab. 9). The sulphur- oxidizing microorganisms were
269 found in eight samples, with the main occurrence in the sand layer with the detection in five
270 cases. The metabolic activity in the both drilling cores was measured with 209 or 220 µW per
271 gram material, respectively. With molecular biological experiments, the differences between
272 samples from drilling cores before and after lowering of the ground water level were found
273 much higher. Extraction of nucleic acids was successful from 30 samples after lowering the
274 ground water and amplification of bacterial 16S rRNA gene in 31 cases. The leaching bacteria
275 *A. ferrooxidans*, *A. thiooxidans* were detected in 18 and 22 samples, additionally
276 *Leptospirillum* spp. was found in 11 cases. Interesting was the detection of leaching
277 microorganisms in the sand and lignite layer, whereas in clay and coarse clay only minor
278 detection rates were obvious (Fig. 6). From the samples before lowering the ground water

279 nucleic acids were extracted successfully in 16 cases and bacterial 16S rRNA gene
280 amplificates were achieved in 5 cases. Specific amplificates for the iron- and sulphur-
281 oxidizing bacteria *A. ferrooxidans*, *A. thiooxidans*, and *Leptospirillum* spp. were not detected
282 in the samples, whereas in two samples amplificates for 16S rRNA genes of *Acidiphilium* sp.
283 were found. A successful amplification of archaeal 16S rRNA genes was achieved in six
284 samples from the sand layer from drilling cores after lowering the ground water level,
285 whereas none amplificate was detected in drilling cores before the ground water lowering.
286

287 **Discussion**

288 In this study the well known leaching microorganisms were detected by cultivation based
289 technique and by amplification and sequencing of the 16S rRNA gene amplificates in the
290 three Lusatian and two mining areas in central Germany. Altogether the *Acidithiobacillus*
291 species were found in the majority of the samples, whereas *Leptospirillum* spp. was found in
292 less quantity (Tab. 10). These findings were also described for mining tailings in Norway or
293 Botswana, respectively (17, 18). Edwards and colleagues (8) found an abundance of *A.*
294 *ferrooxidans* in areas with a pH between 1.5 and 2.3 and ambient temperatures, whereas *L.*
295 *ferrooxidans* was detected at sites with extreme low pH and increased temperatures. *A.*
296 *ferrooxidans* was found in most of the samples and *A. thiooxidans* was detected in lower
297 quantities, this was also described for a single Lusatian lignite mine (31, 32). The distribution
298 of the *Acidithiobacillus* species might be also explained by sand temperatures around 12°C in
299 the overburden and probably the occurrence of psychrotolerant *Acidithiobacillus* spp., as
300 described for *A. ferrooxidans* SS3 (20). The mineral leaching at low temperatures by *A.*
301 *ferrooxidans* was also described for columns or stirred tank reactors (6). Nevertheless, the
302 distribution of the *Acidithiobacillus* species seemed to depend on the mining area. The
303 detection of iron- and sulphur- oxidizing microorganisms by the cultivation based techniques
304 revealed similar results and were supported by the microcalorimetric determination of the

305 microbial activity. This fits to the successful detection of leaching microorganisms from three
306 mine tailings by four different cultivation or molecular based techniques (18).
307
308 In a previous study of mining area B 1 (31) the highest microbial cell counts were found in
309 the freshly liberated overburden, which resulted in an increased metabolic activity, with up to
310 100% microbial activity, shown in this study. Seasonal variations in the microbial occurrence
311 as described before (8) were not found in the lignite mining areas. Despite of the movement
312 of the mining area (B 1) within the two years, the detection of iron- and sulphur- oxidizing
313 microorganisms was not affected. This might be explained by the occurrence of these
314 microorganisms particularly in the sand or lignite layer, as found in the drilling core samples.
315 The isolation and characterisation of moderate thermophilic bacteria e.g. *S. thermotolerans* or
316 *S. thermosulfidooxidans* indicate the occurrence of hot spots in the overburden. These
317 microorganisms were described to grow at low pH and elevated temperatures between 20 to
318 60°C (2). The pyrite oxidation process probably increases the sand temperature and therefore
319 allows the growth of theses microorganisms. The amplification of the archaeal 16S rRNA
320 genes supports these findings, since archaea are known to prefer extreme environments (26)
321 with elevated temperatures above 70°C. Probably due to pyrite oxidation and coal conversion
322 temperatures above 80°C were described for a heap area in Germany (38). Thereby an access
323 of oxygen from three sides of the heap occurred and temperatures up to 90°C were found due
324 to the oxidation processes. In sites with low- grade ore in copper mines sand temperatures
325 between 60 and 80°C, resulting in water temperatures above 30°C, were also described (1).
326 This is supported by the occurrence of archaea only in the sand layer of the drilling cores after
327 lowering the ground water, probably due to a higher porosity of the material. This allows
328 establishing a partial pressure gradient for oxygen and resulting in a gas transfer from air to
329 the sand (37) enhancing the pyrite oxidation.
330

331 The examination of drilling cores after lowering the ground water showed the occurrence of
332 the *Acidithiobacillus* spp. and *Leptospirillum* spp. similar to the findings in the lignite mining
333 areas. In contrast, the cultivation based method showed significantly less signals than the
334 molecular based and might be explained by the technology for the achievement of the drilling
335 cores. During the process the drilling holes were treated with a mixture of water, bentonite
336 and methoxybenzene, which may kill the leaching microorganisms, resulting in a failure of
337 the growth experiments, as reviewed for the effect of hydrocarbons to bacteria (33). The
338 genomic DNA from degraded microorganisms in the overburden is probably stable for up to
339 three years as described for plants (25). Thereby with the molecular based technique a higher
340 detection rate can be explained. Nevertheless, the leaching microorganisms were detected
341 especially in sand and lignite layer. In the clay and coarse clay layer microorganism were not
342 detected, only *A. thiooxidans* was detected once in the coarse clay layer. The reason for this
343 might be an increased supply with oxygen and carbon dioxide due to a better porosity of these
344 materials compared to clay and coarse clay. In the samples from drilling cores before the
345 ground water lowering significant lower detection signals for the leaching microorganisms
346 were found. Only in 4% or 33% of the samples iron- or sulphur- oxidizing bacteria were
347 found in the growth experiments, respectively. The molecular biological based tests revealed
348 only the occurrence of the *Acidiphilium* spp. in two samples, despite of successful extraction
349 of nucleic acids from the samples. Nevertheless, with increasing time of ground water
350 lowering the detection signals for iron- and sulphur- oxidizing microorganisms increased.
351
352 These findings support the proposed idea for the start of the microbial pyrite oxidation. The
353 leaching microorganisms are in the sand but only in low concentrations, therefore no
354 detection was found by cultivation and molecular based technique. Because of the low oxygen
355 content in the ground water these microorganisms are not active. By lowering of the ground
356 water and an influx of air the leaching microorganisms become active and the bioleaching

starts. Because of the limited pyrite content and availability of oxygen in the sand or lignite layer only small quantities of the pyrite were oxidized. By the digging of the lignite and transport to the heap in the open mining areas the microorganisms were mixed in the overburden. Therefore air with oxygen and carbon dioxide, water and pyrite were fully available (37), which results in a maximal (bio) leaching rate. This fits to the observations described before, that a primary pyrite oxidation in open brown coal-mining area proceed by exposition to atmospheric oxygen between section and heap surface. The second stage of pyrite oxidation was described at the surface of recultivated overburden dumps (39, 40). This can be supported by the elementary analysis of a one day sample with higher iron and sulphur content compared to the 30 years old sample. The measurement of sulphur and iron content in the other samples from the mining area B1 support also this result and fit to the decrease in cell number combined with a detection of leaching bacteria with increasing age of the sample as described before (31).

Acknowledgements

We would like to thank Peter Jollas (Mitteldeutsche Braunkohlengesellschaft mbH), Ingolf Arnold and Thomas Koch (Vattenfall Europe Mining AG) and Michael Strzodka (GMB GmbH) for the provision of sand samples and for financial support. Furthermore, thanks are expressed to Jörg Acker and Otto Pulz for the chemical analysis of the sand samples.

We are grateful to Katrin Hentschel and Lisa Maria Domke for technical assistance.

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527 **Figure Legends**

528

529 **Fig. 1.**

530 **Map of Germany with eleven sampling sites of mining areas in Germany. Enlarged**
531 **detail map shows lignite mining areas in Brandenburg (B 1 and B 2), Saxony (S 1 and S**
532 **2), Saxony- Anhalt (A 1), and sampling sites for drilling cores (DC 1 to 6) indicated with**
533 **black arrows.**

534

535 **Fig. 2.**

536 **Photograph of typical sampling sites in the Lusatian open lignite mining area B 1. Hand-**
537 **made 30 cm hole for sampling from freshly liberated overburden exposed to the air for**
538 **one day (A), example for yellow sand incrustations (B), and optically striking drainage**
539 **(C, D).**

540

541 **Fig. 3.**

542 **Sampling site DC 3 near open lignite mining area A 1, after lowering of ground water**
543 **level. Picture A shows the drilling cores from different layers taken from 11.5 to 92.4 m**
544 **depth. In picture B the drilling core from sand layer in 56 to 57 m depth is shown. From**
545 **this layer a sample was taken (depicted with a white arrow) and iron- and sulphur-**
546 **oxidizing microorganisms were detected by growth experiments at 28°C in medium as**
547 **described before (15, 22). Additionally, the species *A. ferrooxidans*, *A. thiooxidans* and**
548 ***Leptospirillum* spp. were identified by molecular biological experiments, as described**
549 **before (5, 21).**

550

551 **Fig. 4.**

552 **Typical agarose gel with nucleic acids separated from humic substances extracted from**
553 **sand samples after electrophoresis through an 0.8% agarose gel. Humic substances were**
554 **visible without staining (left). DNA was visualized with UV (middle) after ethidium**
555 **bromide staining (right).**

556

557 **Fig. 5.**

558 **Scheme of hybridization sites of primer sets on 16S rRNA gene.**

559

560 **Fig. 6.**

561 **Distribution of microorganisms in 42 samples of drilling cores after lowering of ground**
562 **water level from sites DC 3 to 6. From the samples nucleic acids were extracted and**
563 **purified from PCR inhibiting substances. Afterwards detection occurred by PCR**
564 **experiments as described before (4, 5, 21, 23) from the different layers sand (▨), lignite**
565 **(■), clay (▥), and coarse clay (□).**

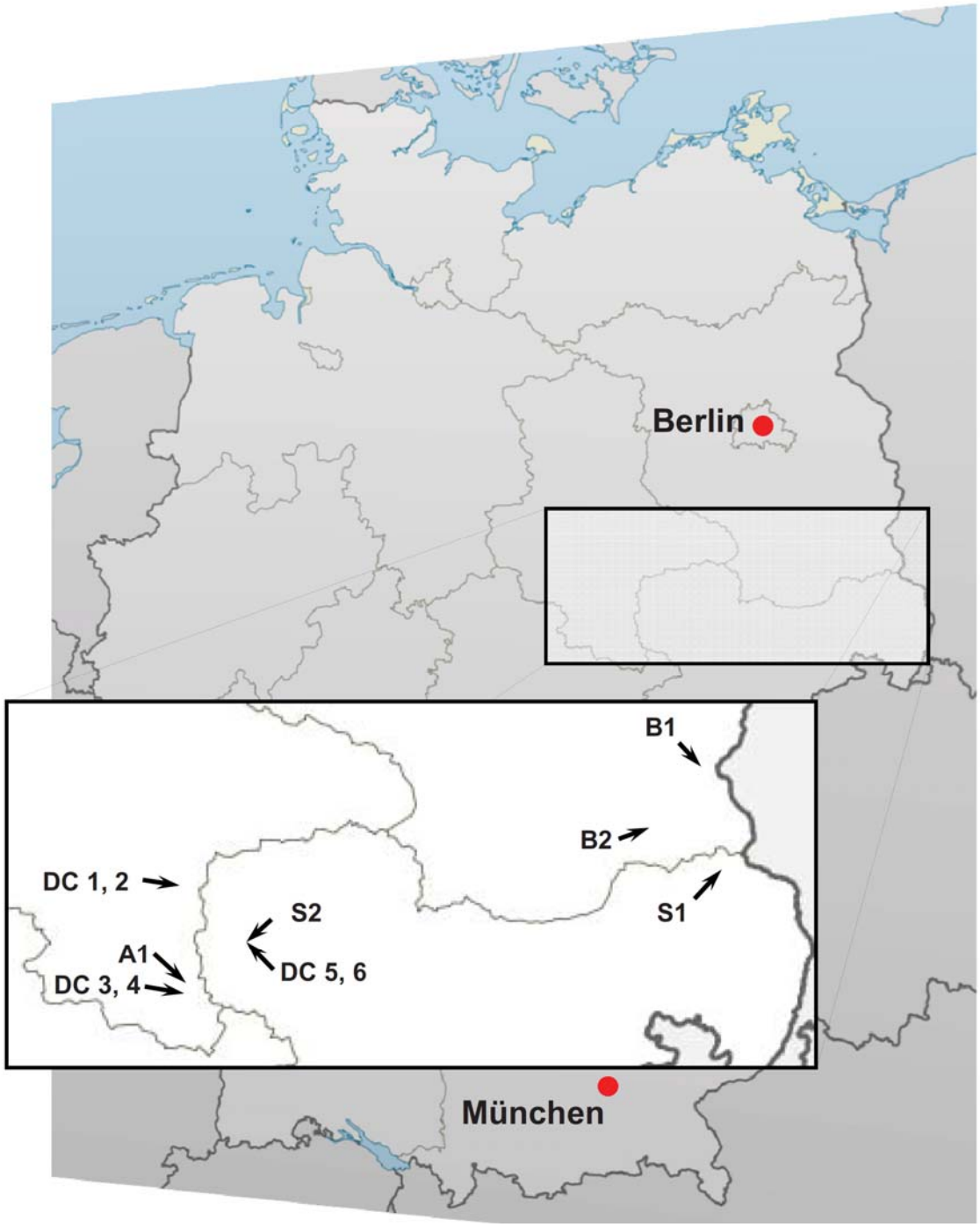


Figure 1

Map of Germany with eleven sampling sites of mining areas in Germany. Enlarged detail map shows lignite mining areas in Brandenburg (B 1 and B 2), Saxony (S 1 and S 2), Saxony- Anhalt (A 1), and sampling sites for drilling cores (DC 1 to 6) indicated with black arrows.

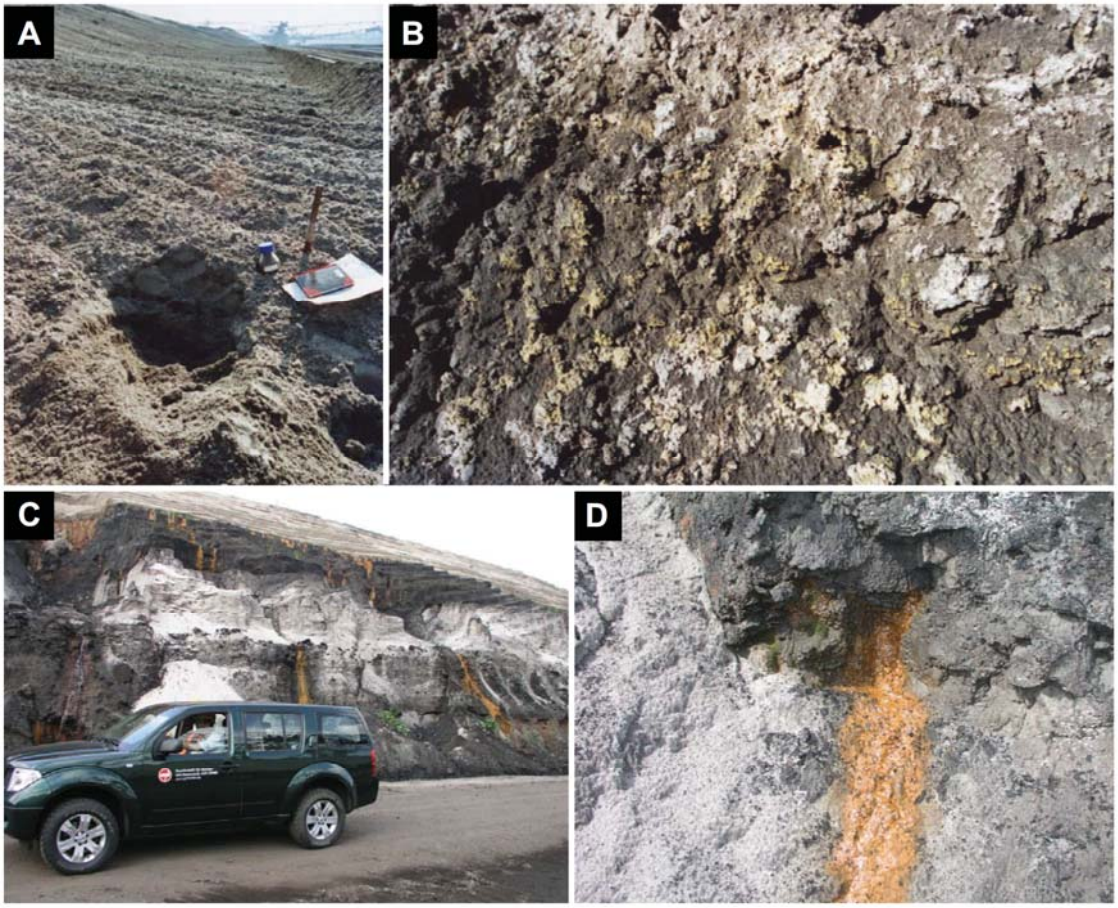


Figure 2

Photograph of typical sampling sites in the Lusatian open lignite mining area B 1. Hand-made 30 cm hole for sampling from freshly liberated overburden exposed to the air for one day (A), example for yellow sand incrustations (B), and optically striking drainage (C, D).

Figure 3

Sampling site DC 3 near open lignite mining area A 1, after lowering of ground water level. Picture A shows the drilling cores from different layers taken from 11.5 to 92.4 m depth. In picture B the drilling core from sand layer in 56 to 57 m depth is shown. From this layer a sample was taken (depicted with a white arrow) and iron- and sulphur-oxidizing microorganisms were detected by growth experiments at 28°C in medium as described before (15, 22). Additionally, the species *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum* spp. were identified by molecular biological experiments, as described before (5, 21).

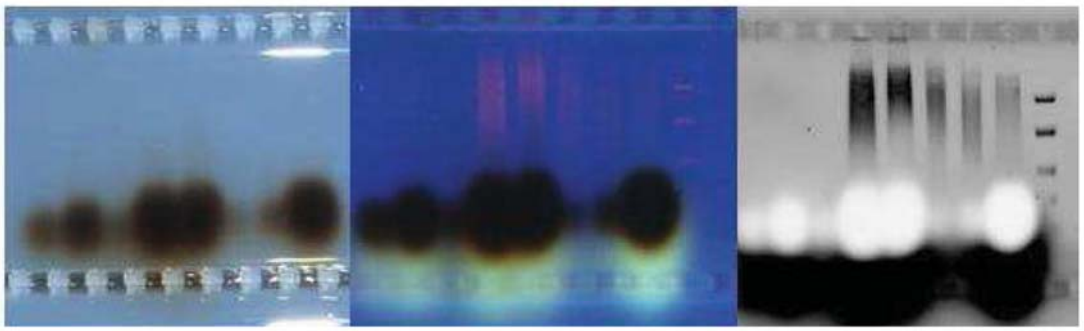


Figure 4

Typical agarose gel with nucleic acids separated from humic substances extracted from sand samples after electrophoresis through an 0.8% agarose gel. Humic substances were visible without staining (left). DNA was visualized with UV (middle) after ethidium bromide staining (right).

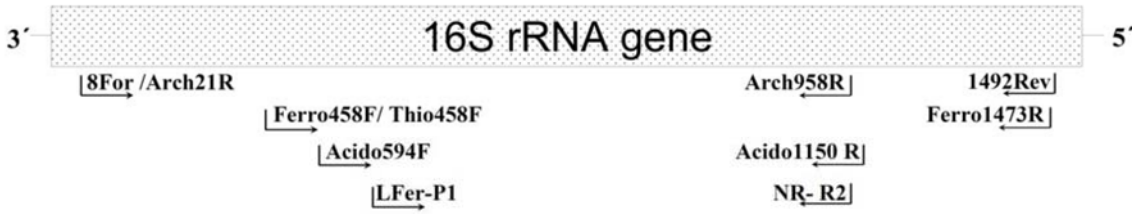


Figure 5

Scheme of hybridization sites of primer sets on 16S rRNA gene.

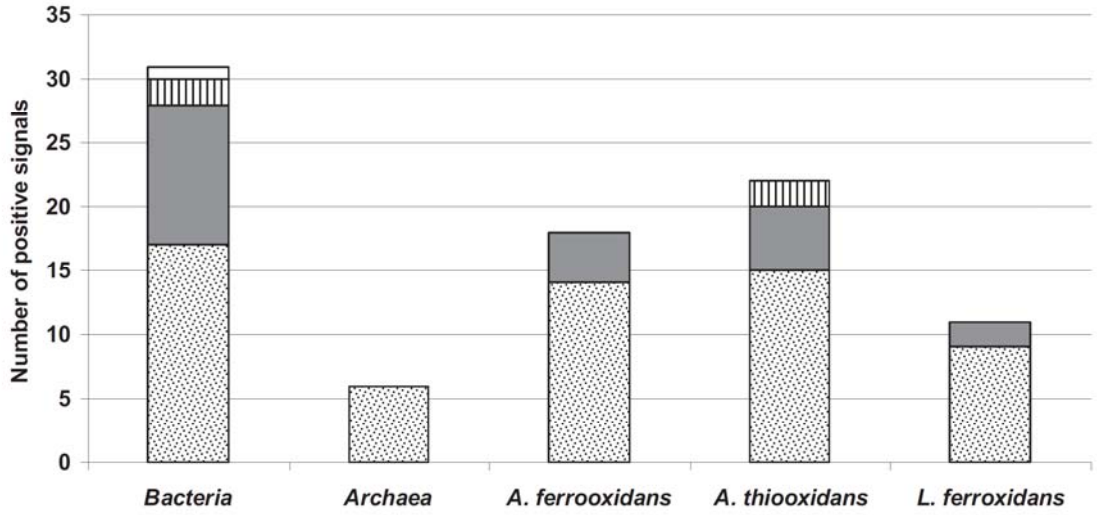


Figure 6

Distribution of microorganisms in 42 samples of drilling cores after lowering of ground water level from sites DC 3 to 6. From the samples nucleic acids were extracted and purified from PCR inhibiting substances. Afterwards detection occurred by PCR experiments as described before (4, 5, 21, 23) from the different layers sand (▨), lignite (■), clay (▥), and coarse clay (□).

Tab. 1.

Sampling campaigns in the lignite mining areas in Brandenburg (B 1 and B 2), Saxony (S 1 and S 2), Saxony-Anhalt (A 1). Sampling sites in the mining area can be found in average 5 km around GPS coordinates. Column four represents the number of samples of the five different sampling areas.

Mining area	Year of sampling	sampling area around GPS Location	Number of samples
B 1	2006	51° 80′N; 14° 50′E	32
	2008		10
B 2	2008	51° 45′N; 14° 62′E	10
S 1	2008	51° 60′N; 14° 20′E	6
S 2	2008	51° 72′N; 12° 38′E	3
A 1	2008	51° 15′N; 12° 19′E	5

Tab. 2.

Sampling of drilling cores (DC) before and after lowering of the ground water level in central Germany, taken in 2008 and 2009. Location of sampling sites is shown by GPS measurement, according to Gauß-Krüger.

drilling cores	year of drilling	year of ground water lowering	Location Height	Location Easting	sampling depth [m]	No. of samples	No. of samples from Sand/ Coarse Clay/Clay /Lignite layer
DC 1	2009	after 2012	80343.0	9233.5	11 – 96	13	9 / 3 / 0 / 1
DC 2	2009	after 2012	80459.9	9119.4	13 – 50	11	5 / 2 / 2 / 2
DC 3	2008	before 2005	81399.5	8000.4	5 – 24	11	7 / 0 / 1 / 3
DC 4	2008	before 2005	78999.9	8445.1	12 – 32	8	3 / 1 / 0 / 4
DC 5	2008	before 2005	64869.8	27007.7	39 – 62	5	1 / 0 / 3 / 1
DC 6	2008	before 2005	64900.0	26800.0	11 – 92	18	7 / 1 / 2 / 7

Tab. 3.

Primer for the amplification of the 16S rRNA genes used in this study.

Specificity	Primer	Sequence 5´to 3´	Reference
Bacteria	8For	AGAGTTTGATCCTGGCTCAG	Meier, 2001 (23)
	1492Rev	GGTTACCTTGTTACGACTT	
Archaea	Arch 21F	TTCCGGTTGATCCYGCCGGA	deLong, 1992 (4)
	Arch958R	YCCGGCGTTGAMTCCAATT	
Leptospirillum spp.	LFer-P1	GGTACTAAGTGTGGGAGGGTTAAAC	Liu *et al*., 2006 (21)
	NR-R2	AGCTGRCGACRRCCATGCA	
A. ferrooxidans	Ferro458F	GGGTTCTAATACAATCTGCT	De Wulf-Durand *et al*., 1998 (5)
A. thiooxidans	Thio458F	GGGTGCTAATAWCGCCTGCTG	
Leaching associated Bacteria	Ferro1473R	TACCGTGGTAACCGCCCT	
Acidiphilium spp.	Acido594F	ACAGTCAGGCGTGAAATTCCTG	
	Acido1150R	AGAGTGCCCACCCAAACAT	

Tab. 4.

Occurrence of iron- and sulphur- oxidizing bacteria in mining area B 1 detected by molecular biological, or cultivation based techniques (see 31, 32), as well as microcalorimetry. Samples were taken at optically striking sites (str) or from the overburden (sys). The results from successful extraction of nucleic acids (DNA) and amplification of 16S rRNA genes specific for bacteria (Bac), as well as for *A. ferrooxidans* (Af), *A. thiooxidans* (At) and *Leptospirillum* spp. (Lf) are reported. Cultivation was performed at 28°C for iron-oxidizing microorganisms (FeoB) or for sulphur-oxidizing microorganisms (SoB) for three to four weeks, as described before (15, 22). Listed are the ratios of positive samples / total samples, or the range of values for microcalorimetry.

Sampling site		Molecular biological detection					Cultivation		activity 1st step	%
B1		DNA	Bac	Af	At	Lf	FeoB	SoB	up to µW	biological activity
2006	sys	24 / 26	23 / 26	20 / 26	8 / 26	6 / 26	18 / 26	22 / 26	0 - 50	5 - 100
	str	4 / 6	3 / 6	3 / 6	2 / 6	3 / 6	6 / 6	5 / 6	1.7 - 30	30 - 90
2008	sys	6 / 6	6 / 6	6 / 6	6 / 6	1 / 6	6 / 6	6 / 6	0 - 62	0 - 100
	str	4 / 4	3 / 4	3 / 4	1 / 4	0 / 4	4 / 4	4 / 4	0 - 261	0 - 100

Tab. 5.

Determination of iron, sulphate, sulphide and calculated pyrite content in samples from Lusatian mining area B 1 taken in 2006. Determination was carried out in February 2007 from Potsdamer Wasser und Umwelt Labor GmbH & Co. KG in Potsdam. Sulphate was determined by procedure DIN EN ISO 10304-D19, sulphide by DIN 4030 and iron by DIN EN ISO 11885-E22. From these data pyrite content was calculated.

sample	No. of samples	Sulphate [mg per kg]	Sulphide [mg per kg]	Iron [g per kg]	Calc. pyrite [mg per kg]
section 1 day	6	522 - 4,588	< 1.5 - 2.8	1.01 - 10.90	< 2.8 - 5.2
section up to 30 years	4	65 - 11,349	< 1.5	0.75 - 9.62	< 2.8
heap 1 day	8	610 - 5,254	< 1.5 - 2.1	0.94 - 4.13	< 2.8 - 3.9
heap up to 30 years	8	33 - 28,741	< 1.5 - 2.6	0.90 - 8.77	< 2.8 - 4.9
optically striking	6	1,487 - 66,448	< 1.5	7.13 - 39.50	< 2.8

Tab. 6.

Determination of sulphur, iron and trace elements in samples from B 1 taken in 2006. Samples represent freshly liberated section or 30 year old heap due to different biological activity, but comparable water content and pH value. Determination was carried out from Institut für komplexe Werkstoffe (IKM) in Dresden. 300 mg of sand were prepared by microwave digestion (μprepA MLS GmbH) and solved in 1 mL HCl/ HNO_3. Suspension was heated to 240°C for 59 minutes, kept at this temperature for 15 minutes and cooled down. Solute was filled up to 50 mL and undiluted SiO_2 was filtered off. Samples were analyzed by ICP-MS (Element XR, Thermo Electron Corporation, United States) for mass range of 6 to 210 and for sulphur and phosphorus by ICP-OES (IRIS Intrepid II XUV, Thermo Electron Corporation, United States) at wavelength 180.71 nm (S) and 177.499 nm (P).

Determined element	Element content [ppm] systematic section 1 day	systematic heap 30 years
Li	6	6
B	3	3
Mg	170	98
Al	3,350	3,300
Na	20	35
Ca	960	750
Ti	18	< 30
Mn	44	17
Zn	6	< 5
Cu	1	0.6
Co	2	0.4
Ni	3	1
Fe	2,250	1,600
Y	2	1
Sr	24	35
Sn	0.3	0.2
Sm	1	< 1
Nd	4	< 5
Ce	6	< 10
La	3	< 5
Ba	12	15
Pb	2	2
Bi	0.3	0.2
Th	1	1
U	0.5	0.4
P	65	70
S	4,000	1,130

Tab. 7.

Detection for iron- and sulphur- oxidizing microorganisms in samples from campaigns in Lusatia (B 2, S 1) and central Germany (S 2, A 1). Samples were taken at optically striking sites (str) or from overburden and heap area exposed to the air for up to 30 years (sys). The results from successful extraction of nucleic acids (DNA) and amplification of 16S rRNA genes specific for bacteria (Bac), as well as for *A. ferrooxidans* (Af), *A. thiooxidans* (At) and *Leptospirillum* spp. (Lf) are reported. Cultivation was performed at 28°C for iron- oxidizing microorganisms (FeoB) or for sulphur- oxidizing microorganisms (SoB) for three to four weeks, as described before (15, 22). Listed are the ratios of positive samples / total samples, or the maximal values in microcalorimetry.

Sample		Molecular biological detection					Cultivation		biological activity
		DNA	Bac	Af	At	Lf	FeoB	SoB	up to µW/g
B 2	sys	4 / 4	4 / 4	4 / 4	4 / 4	2 / 4	4 / 4	4 / 4	53
	str	5 / 6	5 / 6	4 / 6	5 / 6	0 / 6	5 / 6	5 / 6	299
S 1	sys	4 / 6	3 / 6	1 / 6	4 / 6	3 / 6	3 / 6	3 / 6	24
S 2	sys	3 / 3	3 / 3	2 / 3	2 / 3	1 / 3	3 / 3	3 / 3	26
A 1	sys	4 / 4	4 / 4	2 / 4	4 / 4	2 / 4	3 / 4	4 / 4	33
	str	1 / 1	1 / 1	1 / 1	0 / 1	0 / 1	1 / 1	1 / 1	0

Tab. 8.

From drilling core samples after lowering of ground water level from sampling campaigns in 2008 and 2009 (DC 3 to DC 6) molecular biological detection, cultivation for iron- and sulphur- oxidizing microorganisms, as well as microcalorimetric determination was performed. The results from successful extraction of nucleic acids (DNA) and amplification of 16S rRNA genes specific for bacteria (Bac), as well as for *A. ferrooxidans* (Af), *A. thiooxidans* (At) and *Leptospirillum* spp. (Lf) are reported. Cultivation was performed at 28°C for iron- oxidizing microorganisms (FeoB) or for sulphur- oxidizing microorganisms (SoB) for three to four weeks, as described before (15, 22). Listed are the ratios of positive samples / total samples, or maximal values in microcalorimetry.

sampling site	Molecular biological detection					Cultivation		biological activity up to µW/g
	DNA	Bac	Af	At	Lf	FeoB	SoB	
DC 3	10 / 11	10 / 11	10 / 11	8 / 11	1 / 11	0 / 11	0 / 11	125
DC 4	6 / 8	5 / 8	3 / 8	3 / 8	3 / 8	0 / 8	3 / 8	319
DC 5	1 / 5	1 / 5	1 / 5	1 / 5	1 / 5	1 / 5	2 / 5	90
DC 6	13 / 18	15 / 18	4 / 18	10 / 18	6 / 18	1 / 18	7 / 18	29

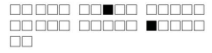
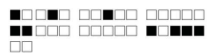
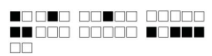
Tab. 9.

Samples of drilling cores before lowering of ground water level from sampling campaigns in 2009 in central Germany (DC 1 and DC 2) were examined by molecular biological detection and cultivation for iron- and sulphur- oxidizing microorganisms as well as microcalorimetric determination. The results from successful extraction of nucleic acids (DNA) and amplification of 16S rRNA genes specific for bacteria (Bac), as well as for *A. ferrooxidans* (Af), *A. thiooxidans* (At), *Leptospirillum* spp. (Lf) and *Acidiphilium* spp. (Ac) are reported. Cultivation was performed at 28°C in medium for iron- oxidizing microorganisms (FeoB) or sulphur- oxidizing microorganisms (SoB) for three to four weeks, as described before (15, 22). Listed are the ratios of positive samples / total samples, or maximal values in microcalorimetry.

sampling site	Molecular biological detection						cultivation		biological activity up to µW
	DNA	Bac	Af	At	Lf	Ac	FeoB	SoB	
DC 1	7 / 13	2 / 13	0 / 13	0 / 13	0 / 13	0 / 13	1 / 13	3 / 13	209
DC 2	9 / 11	3 / 11	0 / 11	0 / 11	0 / 11	2 / 11	0 / 11	5 / 11	220

Tab. 10.

Detection of species in mining areas in Lusatia and central Germany, either by amplification of the 16S rRNA genes directly from sand samples or by isolation of pure cultures and identification by sequencing of 16S rRNA genes in comparison to cultivation at 28°C for iron- and sulphur- oxidizing microorganisms. ■ positively sequenced 16Sr DNA signal, or successful cultivation; □ no signal

Species or Microorganisms	Year / Samples / DNA Source	2006 B1	2008 B1	2008 B2	2008 S1	2008 S2	2008 A1
A. ferrooxidans	Sand						
	Culture						
A. thiooxidans	Sand						
	Culture						
L. ferrooxidans	Sand						
	Culture						
L. ferriphilium	Sand						
	Culture						
F. acidiphilium	Culture						
A. acidophilum	Culture						
A. caldus	Culture						
S. thermosulfidooxidans	Culture						
S. acidophilus	Culture						
S. thermotolerans	Culture						
Iron- oxidizing MO	Culture (28℃)						
Sulphur- oxidizing MO	Culture (28℃)						

4.3.2 Cell lysis of leaching bacteria explains inhibition of pyrite oxidation observed after treatment of sand samples with surfactants

Siebert, H.-M., Y. Wloka, and K.-P. Stahmann

Eingereicht bei Applied and Environmental Microbiology

Cell lysis of leaching bacteria explains inhibition of pyrite oxidation observed after treatment of sand samples with surfactants

Hans-Michael Siebert*, Yvonne Wloka, K.-Peter Stahmann

Fakultät für Naturwissenschaften, Hochschule Lausitz (FH), Großenhainer Str. 57, D-01968 Senftenberg, Germany

Keywords: Acid mine drainage, Bioleaching, Surfactant, Lysis, *A. ferrooxidans*, *A. thiooxidans*, *Ac. cryptum*

Running title: **Inhibition of bioleaching by surfactant-induced lysis**

* Corresponding author, Postal adress: Fakultät für Naturwissenschaften, Hochschule Lausitz (FH), Großenhainer Str. 57, 01958 Senftenberg, Germany. Phone: +49-3573-85-935 / 801. Fax: +49-3573-85-809.

Mailing address: Hans-Michael.Siebert@hs-lausitz.de

Abstract

Iron- and sulphur- oxidizing microorganisms accelerate the oxidation of sulphide minerals, causing a release of sulphuric acid containing products, called acid mine drainage. For countermeasure development the inhibition of leaching microorganisms in columns, filled with overburden material containing 0.4 to 1% pyrite, was performed. The bioleaching was started at temperatures from 12 to 21°C by washing with rainwater. Inhibition was achieved by a single treatment with 2 g/L SDS or Texapon N70 over a period of 38 weeks in 20 mL columns, measurable by sulphate concentrations reduced between 17 and 69% in the leaching solution. In 20 L columns a successful inhibition for up to 45 weeks in the leaching solution and in the sand were found by a sulphate release decreased between 29 and 44%.

For explanation of that inhibition the response of planktonic leaching bacteria to surfactants was compared to reference microorganisms. Inhibition of growth was observed at low concentrations, e.g. 0.005 g/L SDS, for leaching bacteria. At higher concentrations, e.g. 2 g/L, a clearing of planktonic *Acidithiobacillus ferrooxidans* suspensions occurred within seconds suggesting a cell lysis. OD_{600} of high density suspensions decreased by 80% for *A. thiooxidans*, whereas for *Escherichia coli* only a slight decrease was observed after treatment with up to 10 g/L surfactant. Additionally, release of DNA and RNA was found in all bacteria, but protein release was only measured in the leaching bacteria. Labelling with fluorescence stains after surfactant treatment revealed the surviving of *E. coli*, whereas the leaching bacteria lysed.

48 **Introduction**

49 Cell death is a common feature in the live cycle of the microorganisms and has been studied
50 for more than a century (35). The process of cell degradation by several mechanisms is called
51 lysis and linked to the cell wall. It is known that a variety of different factors and enzymes
52 e.g. the peptidoglycan hydrolases called autolysins are involved in this complex process (15,
53 43). These enzymes are involved in the cell division during the separation into mother and
54 daughter cell (13). It was shown that cell lysis of bacteria is essential for the biofilm
55 development (3). In biofilm communities the cell death of a part of the population is linked
56 with a release of macromolecules e.g. proteins and nucleic acids (48). The released genomic
57 DNA can be taken up by the surviving cells (23). The genomic DNA released in the biofilm
58 act also as a structural support (1), it is important for bacterial adhesion (6) and increases the
59 antimicrobial activity (29).
60 It is known that leaching bacteria are able to live in the planktonic state, but are also biofilm
61 developing microorganisms producing a matrix of extracellular polymeric substances for the
62 attachment to the substrate surface (39). Most studies of cell lysis were focussed on model
63 organisms e.g. *Escherichia coli* (24), *Bacillus subtilis* (5), *Streptococcus gordonii* (25) and
64 others, but only a few attempts were performed to understand this process in leaching bacteria
65 (52).
66 The inhibitory impact of substances to the leaching microorganisms was described for the
67 protection of the environment against acid mine drainage (AMD). This is characterized by a
68 contamination of the ground and surface water by solubilised metals (11). Thereby sulphide
69 minerals e.g. marcasite and pyrite are oxidized by biological and chemical processes to
70 sulphuric acid containing products, which are able to solubilise heavy metals from the
71 overburden material. Iron- and sulphur- oxidizing microorganisms were found in mining areas
72 all over the world, mediating and accelerating this process (9, 21, 37). Studies for the
73 inhibition of planktonic leaching bacteria by surfactants were performed for many years with

miscellaneous results, ranging from inhibition (33) to enhancement of the microbial activity (8, 38). But also the treatment with other substances e.g. quaternary ammonium compounds (22, 49) or hydrocarbons (47) was described before. Because leaching bacteria in their natural habitat were found not only in the planktonic state, but also in biofilm communities, surfactant treatment inhibits the adhesion of the bacteria to the mineral surface (31), furthermore a reduced biocide effectiveness was described for *Candida albicans* in the biofilm state (30).

The practical application in large 65 m^3 percolators and successful inhibition of the leaching bacteria and as a consequence the reduction of AMD by 30 to 60 g/L sodium dodecyl sulphate (SDS) and alkaline layers was described before (41). Furthermore, addition of 100 mg/L isothiazolinone, limestone and organic material to the pyritic mine waste resulted in a reduction of up to 50% sulphate or heavy metal release (40). The inhibition of leaching microorganisms by fluorides was described for the pilot scale with 2,000 t low grade ore, resulting in a decrease of the iron extraction rate from 10.4 kg/day down to zero kg/day (14). The reduction of the sulphate oxidation rate by three or four orders of magnitude in uranium tailings or spill material by the treatment with natural phosphate waste was also described (20).

In analysis of a Lusatian mining area the iron- and sulphur- oxidizing bacteria *Acidithiobacillus ferrooxidans* and *A. thiooxidans* were found in the majority of the samples, whereas *L. ferrooxidans* was found only in the minority of the samples (45). With aquifer material from another Lusatian mining area the bioleaching in 20 mL columns was inhibited for 26 weeks by SDS application, measureable by a reduced sulphate release and increase of the pH (46).

The aim of this study was the practical application of surfactants for the inhibition of iron- and sulphur- oxidizing microorganisms in columns filled with overburden material. To clarify

the mechanism of surfactant action, planktonic cultures of the three leaching microorganisms *A. ferrooxidans* ATCC 23270, *A. thiooxidans* DSM 622 and *Acidiphilium cryptum* DSM 2389 were examined in comparison to the two model organisms *E. coli* and *B. subtilis* and a model of surfactant induced cell lysis was proposed.

Material and Methods

Surfactants

In this study the anionic surface active compounds Texapon N70® (sodiumlaurylethersulphate) supplied by the Cognis GmbH (Düsseldorf, Germany) and sodium dodecyl sulphate (SDS) were tested. SDS was purchased as 99% active substance (Carl Roth GmbH, Germany), whereby Texapon N70 consisted of 68 to 73% active substance. The surfactants were diluted in deionised-water to 10% (weight/volume). For the column experiments the surfactants were diluted with collected rainwater to appropriate concentrations.

Maintenance and inhibition experiments with reference cultures

The growth inhibition and lysis experiments with planktonic cultures were carried out with five reference microorganisms in test tubes or 96-well plates. The leaching bacteria *A. ferrooxidans* ATCC 23270 and *A. thiooxidans* DSM 622 were cultured in modified basal salt media according to Mackintosh (26) at 28°C containing 1 mM ammonium chloride instead of ammonium sulphate and additional 5 g/L elementary sulphur instead of ferrous sulphate. *Ac. cryptum* DSM 2389 a heterotrophic bacterium was cultivated in liquid media with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) designation 269 at 28°C.

For comparison the Gram-negative bacterium *E. coli* DH5α containing the plasmid pAG203 *GLY1* (28) was routinely grown at 37°C in Luria-Bertani medium containing 100 µg/mL Ampicillin.

As a further control, Gram- positive *B. subtilis* was maintained in PME Medium with DSMZ designation 1 at 28°C.

Inhibition experiments were performed with SDS or Texapon N70 in media as described above. For experiments in logarithmic growth phase 500 µL bacterial suspension was transferred in five millilitre fresh media and additional 500 µL surfactant dilutions were added to final concentrations of 0.001 to 10 g/L. Test tubes were incubated for different periods according the growth rates from seven to 20 days and analysed for growth by determination of the turbidity of the culture broth. For analysis of inhibition in the stationary phase bacterial cultures were cultured to mid-logarithmic phase in media as described above. Bacterial suspensions were transferred into test- tubes and equal surfactant dilutions were added to final concentrations of 0.001 to 10 g/L. The growth inhibition was analysed by clearance of the medium after incubation for two days to one week, according to bacterial growth rates.

Cell lysis kinetics

Suspensions of reference microorganisms were centrifuged for up to 30 minutes at 14,800 x *g* and the supernatant was removed. The pellet was suspended in 0.9% sodium chloride and adjusted to an optical density of 0.6 to 0.8 at a wavelength of 600 nm (OD_{600}). A volume of 900 µL of this concentrated cell suspension was transferred into cuvettes. After 100 seconds of equilibration in the photometer, 100 µL of surfactant dilution were added to final concentrations of 0.001 to 10 g/L. The OD_{600} was measured continuously for 900 seconds at 28°C.

Protein and nucleic acid release

Followed by determination of the optical density after surfactant treatment, bacterial suspensions were centrifuged for 20 minutes at 13,000 x *g*. The cell pellets were used for determination of nucleic acid release. The supernatant was divided in two parts, for determination of nucleic acid release or release of proteins.

For analysis of nucleic acid release triggered by surfactant, the cell pellets were resuspended in 25 µL water. Nucleic acids in 500 µL of supernatant fraction were precipitated with an equal volume of isopropanol and 20 µL 5 M sodium chloride. After 30 minutes incubation at -21°C, the samples were centrifuged for 15 minutes at 20,000 x *g* and the supernatant was discarded. The pellet was washed twice with 70% ethanol, dried at 45°C and resuspended in 12.5 µL water. For visualisation of nucleic acids, equal amounts of pellet and supernatant fraction were separated by electrophoresis on 0.8% agarose gels and stained with ethidium bromide (10 µg/mL). For differentiation between DNA and RNA release, nucleic acids were treated with either 1 Unit of DNase I or RNase for four hours at 37°C. *Eco*RI digested plasmid Yep352-p25 (Maeting *et al*., 1999) was used as DNase substrate.

For protein determination the supernatant was diluted to appropriate concentrations with deionised water. This was used for measurement of the protein content with Micro BCA™ Protein Assay Kit (No. 23235, Thermo scientific, Germany) in accordance with the manufacturer's instruction and standardized to bovine serum albumin. For determination of the total protein content, *E. coli* cells were ground to a powder in liquid nitrogen followed by a protein determination. The content per cell was calculated and used as reference for all other microorganisms to calculate the total amount of protein per cell.

Viability staining

The survival control after surfactant treatment was performed with LIVE/DEAD BacLight bacterial viability kit (No. L-13152 Invitrogen, Germany), according to the manufacture's

175 instructions. The protocol for dead or alive controls was adapted to the work with leaching
176 microorganisms by adjusting volumes of bacterial suspension and centrifugation conditions.
177 For preparation of SDS treated cells two millilitres cell suspension was centrifuged for 15
178 minutes at 10,000 x *g*. The pellet was resuspended in 0.9% sodium chloride and mixed with
179 SDS to final concentrations from 0.25 to 10 g/L. After 15 minutes of incubation, the
180 suspension was centrifuged at 10,000 x *g* for 15 minutes and the pellet was resuspended in 1
181 mL filter-sterilized deionised-water. Either 60 µL bacterial suspension with living, dead or
182 SDS treated cells, were mixed with 30 µL of SYTO9 and equal volume propidium iodide
183 (PI). The final dye concentrations were 6 µM SYTO9 and 30 µM PI. Mixtures were incubated
184 in the dark for 15 minutes and afterwards analyzed by fluorescence microscopy with an
185 Axioscope 2 plus equipped with a camera Axiocam HRC (Zeiss, Germany). Viable cells were
186 observed with the Zeiss filter set 13 (excitation wavelength, 450- 490 nm; emission
187 wavelength, >515 nm) and not viable cells were monitored with the filter set 05 (excitation
188 wavelength, 470- 490 nm; emission wavelength, >635 nm).
189
190 Fluorescence spectrometry of the cell suspensions treated with SYTO9 and PI was performed
191 with Fluoromax-4 Spectrofluorometer, Horiba Jobin Yvon (Bensheim, Germany) and an
192 emission spectrum between 490- 700 nm fluorescence of each cell suspension was measured
193 with an excitation wavelength of 470 nm and recorded.
194
195 **Sampling and mineralogical analysis**
196 In June 2009 a sampling campaign in an open lignite mining area in Lusatia (51° 45´N; 14°
197 62´E) was performed. From the overburden one ton aquifer material was sampled in the main
198 working level and a depth of 72.4 to 72.7 meter above sea level. To minimize the external
199 influence the upper 50 cm sand layer was removed (Fig. 1A). Afterwards plastic liner with
200 one meter length and around 10 cm diameter were pressed in the sand layer and pulled- out

201 with the sand samples (Fig. 1B to D). The air was removed from the unaffected material by
202 the addition of around 50 g dry ice pellets and sealing of one end of the liner. After the gas
203 emission the liners were sealed air tight and the material was stored at 20°C.
204 From the sand material two independent mineralogical analyses were performed by X-ray
205 diffraction. The analyses in 2009 and a second analysis in 2011 were performed with material
206 of a mixture from three liners.
207
208 **Leaching experiments in columns**
209 The application of the surfactants SDS and Texapon N70 was performed in columns in 20 mL
210 and 20 L scale (Fig. 2). The experiments in 20 mL scale were carried out as described (46).
211 The inhibition experiments in 20 mL scale were performed with SDS, Texapon N70, or a 1:1
212 mixture of both surfactants. The surfactants were solubilised in rainwater to final
213 concentrations of 2 g/L or 1 g/L for the surfactant mixture. Furthermore, the experiments were
214 performed at three different temperatures 21°C, 15 to 21°C or at constant 12°C. For the
215 experiments at 21°C additional control columns were operated with e.g. addition of iron- and
216 sulphur- oxidizing bacteria.
217 Experiments in 20 L scale were performed in columns filled with 12.0 to 13.6 kg (wet weight)
218 aquifer material. Three independent runs were performed with two columns in parallel. One
219 was set as the control column washed only with rainwater and the second column, first
220 washed with rainwater and than treated with the surfactant solution. For the inhibition
221 experiments SDS or Texapon N70 were solubilised in rainwater to final concentrations of 2,
222 10, or 60 g/L. Two runs were carried out at 15 to 25°C, and a third run was performed at
223 constant 12°C. The control columns were started with a single addition of $2.6x10^{10}$ to
224 $5.2x10^{11}$ cells suspended in one litre basal salt medium (pH 2) according to Mackintosh (26)
225 in the first run, or in one litre rainwater in the second and third run. The suspension consisted
226 of a mixture of *A. ferrooxidans*, *A. thiooxidans*, *L. ferrooxidans*, and *L. ferriphilum* grown to

mid-logarithmic phase in the adequate medium (16, 26) at 28°C and harvested by centrifugation at 22,000 x *g* for 30 minutes. The leaching in the other column was started by the addition of one litre rainwater. The leaching solution of the columns was collected and loaded every three days onto the columns. In the first run the leaching solution of both columns was changed three times by one litre fresh rainwater every 7 to 15 days. In the second and third run the leaching solution was changed after 15 to 24 days by one litre fresh rainwater. The surfactant treatment was started in the first run after 11 weeks, in the second and third run after five weeks. Therefore Texapon N70 was solubilised in one litre rainwater to final concentrations of 2 or 60 g/L and loaded onto the columns. The surfactant solutions were loaded again when the complete volume run through the columns. The surfactant solutions were changed after a period between one and six weeks. After 38 weeks in the first run the surfactant treated column was emptied, than the aquifer material was filled again in the column, together with 50 gram SDS solubilised in five litres rainwater. The columns were operated between 33 and 52 weeks per run.

During the three different runs measurement of the pH value was performed every week. Determination of the sulphate content of the leaching solution was performed in double from every leaching solution with Spectroquant® test (No. 1.14791.0001; Merck, Germany), following manufacturer's instructions. Additionally, the content of ferrous and total iron in the leaching solution was measured with Spectroquant® test (No. 1.00796.0001; Merck, Germany), following manufacturer's instructions. The content of ferric iron was calculated as difference of total and ferrous iron.

For the study of the surfactant induced change in cell count of the acidophilic iron- and sulphur- oxidizing microorganisms in the leaching solution or in the aquifer material the most-probable- number- technique (MPN- technique) was used. Microorganisms were cultivated in liquid media containing 10 g ferrous sulphate per litre (26) and in media described by Hutchinson *et al*. (16) with 10 g elementary sulphur per litre. The cultures were

253 incubated in 96- well plates or test tubes for three to four weeks at 28°C. Sand samples were
254 analysed before the experiment, during surfactant addition to the column and at the end of the
255 experiment. The leaching solution sampling was additionally performed after the rain water
256 change.
257
258 **Results**
259 **SDS or Texapon N70 reduced bioleaching in columns filled with overburden material**
260 For an application of the surfactants to planktonic as well as cells growing as a biofilm on the
261 mineral surface, experiments in 20 L columns were performed with aquifer material. The
262 material was analysed for the content of pyrite or marcasite. In two independent analyses
263 pyrite contents between 0.4 and 1% were found. The iron sulphide marcasite was not detected
264 in the samples. The aquifer material was filled into the columns, containing 10^5 to 10^6 cells
265 per gram sand sulphur-, or 10^4 to 10^5 iron- oxidizing microorganisms per gram sand.
266 In the first run, performed at 15 to 21°C, the leaching solution was tested for iron- and
267 sulphur- oxidizing microorganisms by the MPN- technique. These microorganisms were
268 detected in the leaching solution of the control column in a range from $3x10^4$ to $3x10^6$ cells
269 per millilitre (Fig. 3). After two weeks $4x10^8$ cells per millilitre were detected, due to the
270 addition of iron- and sulphur- oxidizing microorganisms to the column. In the leaching
271 solution of the other 20 L column equal cell counts were found until week 14. Afterwards the
272 cell counts dropped below the detection limit of 30 cells per millilitre until week 24, due to
273 the Texapon N70 treatment. The pH of the leaching solution of the control column washed
274 with rainwater dropped within five weeks from pH 6.8 to 2.5 and remained stable between pH
275 2 and 2.5 until week 50. In the leaching solution of the other 20 L column a pH similar to the
276 control column was measured. Four weeks after the Texapon N70 treatment an increase to pH
277 3.5 was measured and remained stable until week 30. Determination of iron content and
278 calculation of ferrous to total iron ratio supported the inhibition of leaching microorganisms

with the occurrence of 99% ferrous iron in comparison to maximal 10% in the control column. Until week 36 the cell counts of the iron- and sulphur- oxidizing microorganisms increased, resulting in a decreasing pH of 2.5 until week 38, but after SDS treatment leaching microorganisms were not detected in the leaching solution or in the sand samples. Due to the SDS treatment in week 38 the pH of the leaching solution increased again to pH 3.5. The determination of the ferrous to total iron ratio supported the successful inhibition of leaching microorganisms by a measurement of 98% iron in the reduced form. The inhibition of iron- and sulphur- oxidizing microorganisms due to the surfactant treatment was additionally supported by the continuous measurement of the sulphate release. In week 50 the sulphate concentration in the leaching solution was measured minus 87% compared to the rainwater washed control. Over the complete running time the cumulated sulphate release was minus 29% compared to the control.

In the second and third run with the 20 L columns the cell counts of iron- and sulphur-oxidizing microorganisms of the leaching solution in the control columns were detected in the concentration range similar to the first run for the entire running time. The pH value of the leaching solution decreased within this time from pH 2.5 or 3.5 after two weeks below pH 2 at the end of the experiment in the second or third run, respectively. Cell counts of the iron-and- sulphur- oxidizing microorganisms in the leaching solution of the surfactant treated columns were equal to rainwater washed controls, but dropped to below one cell per millilitre, direct after the Texapon N70 treatment and remained stable until the end of the runs. Additionally, growth experiments for iron- and sulphur- oxidizing microorganisms with the sand samples taken from different column depths revealed no living leaching microorganisms too. Due to the surfactant treatment the pH value in the second run increased from pH 2.5 to pH above 2.7 and remained in this range until the end. In the third run performed at constant 12°C the pH increased due to the Texapon N70 application from pH 3.3 to a stable pH above 4.3. The successful inhibition of the leaching microorganism was also indicated by the

305 determination of the ratio of ferrous to total iron in the leaching solution (Fig. 4) in second
306 and third run.
307 The second and third run operated at 15 to 21°C and at constant 12°C showed inhibition in a
308 similar way, indicating an independence of the operating temperature. An inhibition of minus
309 34% or minus 44% concerning total sulphate release was measured due to treatment with
310 Texapon N70 (Fig. 5). Additionally, the sulphate concentration in the leaching solutions at the
311 end of the experiments were measured minus 68% or minus 72% compared to the controls in
312 the second or third run, respectively.
313
314 **Scale down from 20 L to 20 mL columns showed consistent effect of surfactants on**
315 **bioleaching**
316 For a scale down experiments in 20 mL columns were performed (Fig. 2). The columns were
317 filled with 16 to 17 gram of the aquifer material that had been used for the 20 L columns. The
318 sand contained 10^5 to 10^6 cells per gram sand sulphur-, or 10^4 to 10^5 iron- oxidizing
319 microorganisms per gram.
320 In the leaching solution of the control columns washed with rainwater the cell counts for iron-
321 and sulphur- oxidizing microorganisms were found in a range between 10^4 and 10^6 cells per
322 millilitre for the complete run of 36 weeks. Significant differences in the cell counts of iron-
323 and sulphur- oxidizing microorganisms of the control columns due to the operating
324 temperatures between 12 and 21°C were not found (Fig. 6A). Single applications of the
325 surfactants resulted in a complete disappearance of iron- and sulphur- oxidizing
326 microorganism over the complete run of 36 weeks at 12 to 21°C. A detection of iron- and
327 sulphur- oxidizing microorganisms was positiv after 16 weeks in the column with Texapon
328 N70 application, operated at 15 to 21°C. At the end of the experiments the columns were
329 emptied and the sand was tested for iron- and sulphur- oxidizing microorganisms. In the
330 rainwater washed columns between 10^4 and 10^7 iron- or sulphur- oxidizing microorganisms

per gram sand were detected at all three temperatures. No growth was detectable when the sand of the surfactant treated columns was investigated by the MPN method. The pH values of the leaching solution of the rainwater washed control columns started at 4 to 4.5 and decreased to values around pH 2.5 in week 23. At the end of the experiments a further decrease of the pH to values between pH 1.7 and 2.1 was measured in the leaching solution. The pH value of the leaching solution of the surfactant treated columns was 4.5 at the beginning of the experiments. In the leaching solution of the columns operated at 21°C a decrease to pH 2.3 to 2.7 was found. In the leaching solution of surfactant treated columns at 15 to 21°C or constant 12°C the pH value was stable to week 30 between pH 3.1 and 3.8 and decreased to pH 2.7 to 3.4 at the end of the experiments. Furthermore, the measurement of ferrous iron and total iron content was measured from the leaching solution of the columns at the end of the experiments and the ferrous iron content was calculated. In the rainwater washed control columns the ratio between ferrous and total iron was found between 1 and 6% at the end of the experiment, independent from the temperature. In the leaching solution of the surfactant treated columns a ratio between 91 and 99% was found, indicating a successful inhibition. The sulphate release of the 20 mL columns into the leaching solution was also determined at regular time points. The successful leaching in the columns washed with rainwater at 21°C was measurable by a stable sulphate release at 2 g/L for 11 weeks, than increasing to concentrations above 8 g/L until week 38 (Fig. 6B). The same behaviour was measured for the control columns operated at 15 to 21°C or at constant 12°C. At temperatures from 15 to 21°C lower sulphate concentrations were found and the maximum of sulphate release with 8 g/L occurred in week 38. In control columns operated at constant 12°C lower sulphate concentrations in the leaching solutions were measured over the complete 38 weeks and the increased sulphate release occurred from week 30 with 3 g/L. The columns with a single surfactant treatment at the three different temperatures showed lower sulphate concentrations over the complete 38 weeks, beside of Texapon N70 treated column at 15 to

21°C in week 18. Compared to the water rainwater washed control the surfactant treated columns at 21°C released between 62 to 67% less sulphate. In column experiments performed at 15 to 21°C between 42 and 69% and at constant 12°C from 17 to 64% less sulphate were released, due to a single surfactant treatment.

Growth inhibition and clearance of bacterial suspensions

For an analysis of the inhibition of the bioleaching in columns the surfactant induced stress response of four Gram- negative reference bacteria in planktonic cultures at different surfactant concentration was investigated. The growth of planktonic bacteria was tested in tubes. Cultivation of bacteria in media mixed with surfactant, resulted in a decreased or no turbidity depending on the concentrations of surfactant used (Fig. 7). The leaching bacteria showed a higher sensitivity to surfactant treatment than *E. coli* (Tab. 1). Treatment with SDS or Texapon N70 in logarithmic growth phase resulted in minimal inhibitory concentration of 500 mg/L for *E. coli*. For the growth inhibition of e.g. *A. ferrooxidans* only 1% of the surfactant concentration was necessary, in comparison to *E. coli*. Because the phenomenon of clearance of the bacterial suspension was visible, the ability of surfactants to trigger cell lysis was determined. Therefore pre- cultivated stationary phase cultures were transferred to medium mixed with surfactant solution. It was found that higher surfactant concentrations were necessary (Tab. 2). Visible was a clearance of the bacterial suspension after addition of the surfactants. These results led to the question whether a time course of clearance can be followed by detection of the extinction at 600 nm.

Surfactants caused a clearance of bacterial suspensions within seconds

Treatment with the surfactants resulted in a clearance measurable as time course of decrease in optical density. Depending on the microorganism or used surfactant with 10 g/L different kinetics were observed (Fig. 8). Suspensions of *B. subtilis* or *E. coli* showed the weakest

383 sensitivity of all tested microorganisms (Fig. 8). For these microorganisms the OD_{600}
384 decreased 15 to 25% for SDS concentrations from 0.5 to 10 g/L. In contrast, the OD_{600} of the
385 leaching bacteria *Ac. cryptum* and *A. ferrooxidans* treated with the same concentration regime
386 of SDS showed a decrease of 50 to 60% after 300 seconds. The strongest response to
387 surfactant treatment was found for *A. thiooxidans* with a decrease of even 70 to 80%. Higher
388 concentrations of SDS (60 g/L) resulted in no stronger decrease. Treatment of *A. thiooxidans*
389 or *A. ferrooxidans* suspensions with low SDS concentrations from 0.025 to 0.25 g/L resulted
390 in an increase of the optical density for up to 35%.
391 The response of the bacteria was also tested with the surfactant Texapon N70 under the same
392 experimental conditions. All four microorganisms showed a slower decrease of OD_{600}
393 compared to the treatment with the same SDS concentrations. The weakest sensitivity with a
394 decrease of 11% at a concentration of 10 g/L surfactant was found for *E. coli*.
395
396 **Surfactant triggered release of macromolecules**
397 The decrease within seconds in optical density suggested a lysis mechanism triggered by the
398 surfactant treatment. Therefore the release of the nucleic acids after surfactant treatment
399 monitored by optical density was investigated for *E. coli* and the three leaching associated
400 bacteria.
401 All four species showed a surfactant triggered release of nucleic acids. In the supernatant of
402 SDS treated *E. coli* suspensions nucleic acids with a size below 850 bp were visible for
403 concentrations from 1 to 5 g/L, whereas from *Ac. cryptum* suspension nucleic acids were
404 liberated at concentrations above 0.25 g/L SDS (Fig. 9). In the pellet fraction of both
405 microorganisms nucleic acids with a size above 10 kb were found for SDS concentrations
406 down to 0.25 g/L. Additionally, concentrations of 0.25 or 0.5 g/L SDS caused a release of
407 nucleic acids with a size below 850 bp for *E. coli* or *Ac. cryptum*, respectively. Treatment
408 with Texapon N70 resulted also in a release of high as well as low molecular weight nucleic

409 acids. The surfactant concentrations required for an effect were in a similar concentration
410 range compared to the SDS triggered response (Fig. 9). Furthermore, from the *A. ferrooxidans*
411 suspensions nucleic acids with a size above 10 kb were liberated in the pellet fraction at SDS
412 concentrations above 0.05 g/L, additionally a smear was obvious at 0.025 g/L. For Texapon
413 N70 the liberation of high molecular weight nucleic acids in the pellet fraction was detected
414 by treatment with concentrations above 0.25 g/L (Data not shown). The SDS caused
415 liberation of nucleic acids from *A. thiooxidans* was shown before (46). The treatment with
416 Texapon N70 concentrations above 0.25 g/L caused a release of high molecular weight
417 nucleic acids above 10 kb, as well as nucleic acids below 1 kb.
418 To distinguish between DNA and RNA release the pellet fraction of *E. coli* or *Ac. cryptum*
419 suspensions after treatment with 10 g/L SDS or Texapon N70 was additionally investigated
420 after DNase or RNase incubation (Fig. 10). In both microorganisms DNA was released above
421 10 kb and with a smaller size below 2 kb, triggered by the surfactant. The surfactant caused
422 released RNA had a size below 2 kb.
423
424 Since surfactants triggered the release of DNA and RNA, the liberation of proteins was
425 investigated. Therefore the amount of protein in the supernatant was measured after SDS
426 addition and time course measurement of the optical density in the photometer. For
427 comparison *E. coli* cells were ground to a powder in liquid nitrogen followed by protein
428 determination. A total amount of 200 fg protein per cell was calculated. This reference was
429 used for all other microorganisms to calculate the total amount of protein per cell. The
430 treatment of *E. coli* suspensions with concentrations above 1 g/L resulted in a release of 0.2 to
431 0.35% of total protein in the supernatant and a decrease to 0.1% at 10 g/L. For the three
432 leaching bacteria an increasing release of protein at concentrations above 0.25 g/L SDS was
433 measured (Fig. 11). For the heterotrophic *Ac. cryptum* a first protein release was measurable
434 at 0.25 g/L SDS with 15% of the total protein. Concentrations above 0.5 g/L SDS caused a

435 release of 45% of the total protein. The iron- and sulphur- oxidizing bacteria *A. ferrooxidans*
436 and *A. thiooxidans* showed liberation of the protein at concentrations from 0.25 to 0.5 g/L
437 SDS. Thus 65 or 80% of the protein of *A. ferrooxidans* or *A. thiooxidans* were measured,
438 respectively.
439
440 **Survival of the bacteria after surfactant treatment**
441 To answer the question whether SDS triggered release of nucleic acids and proteins can be
442 explained by membrane permeabilization viability staining was performed. The *E. coli* and
443 *Ac. cryptum* cells were grown to logarithmic phase, treated with SDS and a staining with
444 SYTO9 and PI was performed, to ascertain cell viability. The living, isopropanol- killed and
445 SDS treated cells were analysed with fluorescence microscopy by stimulating the fluorescent
446 stains SYTO9 or PI separately and the pictures were merged. Both bacteria showed for the
447 living cells green fluorescence, indication of an intact membrane status (Fig. 12). The cells
448 incubated in isopropanol showed a yellow colour, the overlap of green and red, indicating a
449 membrane permeabilization, that PI can enter the cell by the damaged cytoplasmic
450 membranes. SDS treatment of *E. coli* cells showed only red or green fluorescence similar to
451 the alive control, but with a higher amount of red fluorescence cells. The *Ac. cryptum* cells
452 treated with SDS showed only yellow colour, similar to the isopropanol- killed control
453 indicating a damaged membrane status. Furthermore, shrinkage of the cells after surfactant
454 treatment was observed for all tested bacteria.
455
456 Additionally to the different fluorescence microscopy pattern of *E. coli* and *Ac. cryptum* after
457 SDS treatment, spectrometric analysis was performed with *E. coli* and the three leaching
458 bacteria. The bacteria were treated with different SDS concentrations and fluorescence
459 spectrometric analysis was performed (Fig. 13). Interesting was the different behaviour of *E.*
460 *coli* in comparison with the leaching microorganisms. All four bacteria showed for living cells

461 a strong signal in the area around 510 nm, indicating an intact membrane. The analysis of the
462 isopropanol- killed cells of the three bacteria (not performed for *Ac. cryptum*) showed signals
463 in the area around 630 nm, indicating a damaged cytoplasmic membrane. *E. coli* showed with
464 increasing SDS concentrations a decreasing live signal at around 510 nm. But increasing SDS
465 concentrations did not lead to an increasing signal at 630 nm (Fig. 13A). The leaching
466 bacteria in contrast showed complete different fluorescence pattern. With an SDS
467 concentration of 0.25 g/L a high signal at around 630 nm was found, in all three cases higher
468 than the signal for the isopropanol- killed cells. Furthermore, the three bacteria showed only a
469 weak or no increased signal for living cells at 510 nm. Increasing SDS concentrations lead to
470 a decreasing signal around 630 nm, even below the signal for the dead cells (Fig. 13B to C).
471
472 **Discussion**
473 In this study the inhibition of bioleaching by SDS and Texapon N70 was shown. Furthermore,
474 this phenomenon was explained by a triggering of cell lysis for some relevant bacterial
475 species in pure planktonic culture.
476
477 **Tenside- triggered cell lysis**
478 The time course measurement in the photometer with a decrease in the OD_{600} at high
479 surfactant concentrations suggested a lysis or degradation of the cells, as described before for
480 several other bacteria (10, 24, 34). The increase of the optical density at low surfactant
481 concentrations is probably caused by a swelling of the cells as a first response of the bacteria,
482 as described in mitochondria (18, 19). This observation was also supported by the
483 microscopic observation of a change in cell shape and a decrease of fluorescence
484 spectrometry signal intensity at around 510 nm after the surfactant treatment.
485 The autolysis of microorganisms is accompanied with the release of nucleic acids and
486 proteins. It was shown in this study that three leaching bacteria and *E. coli* showed a

surfactant triggered release of DNA and RNA. The release of nucleic acids due to an external stimulus was described for e.g. *Streptococcus* spp. and *Enterococcus faecalis* (6, 23, 50). For the iron- and sulphur- oxidizing bacterium *A. ferrooxidans* the nucleic acid release caused by organic acids was also described before (52). It was shown that the released nucleic acids consisted of chromosomal DNA with a size above 12 kb, as well as from RNA with a lower molecular weight (23). The release of nucleic acids from the bacteria at high surfactant concentrations were found with a molecular size above 10 kb in this study. Because no degradation was found after agarose gel electrophoresis, the release of chromosomal DNA can be concluded, but has to be checked. The release of nucleic acids was described to support the biofilm development (1), the adhesion of the microorganisms to the substrate surface (6), is important in the initial phase of the biofilm development for e.g. *Staphylococcus epidermis* (34). Since the three leaching bacteria are known to live in a biofilm state, the release of nucleic acids might promote the attachment to the mineral surface, e.g. pyrite. The autolysis of a part of the population, which is accompanied with the release of DNA and the development of competence, might be an evolutionary advantage as described for *Streptococcus pneumonia* (48). The gene transfer to the surviving major part of the bacterial community may increase the antimicrobial activity, as described for *Pseudomonas aeroginosa* (29). This was supported by the growth experiments, as well as by the labelling with fluorescent dyes, where the leaching bacteria lysed, whereas the *E. coli* was found alive after surfactant treatment. The low protein release after the surfactant treatment measured for *E. coli* was probably due to the removement of the outer membrane surface, or by unspecific interaction of the surfactants with the cell membrane (17) causing a decomposition of the cytoplasma membrane (12). Probably, only a small part of the community undergoes lysis, which supports the idea of competence development. In contrast, the lysis of the leaching bacteria released between 50 to 80% of the intracellular proteins which supports the idea of death and lysis of the major part of the community as found by the fluorescence spectrometry.

513 These results suggest a similar way of action of the surfactants SDS and Texapon N70. The
514 differences in the susceptibility of the leaching bacteria to the surfactants in comparison to *E.*
515 *coli* might be explained with differences in the activation or inactivation of the autolysins. For
516 the bacteria *E. coli* and *B. subtilis* the treatment with surfactants caused the autolysis of only a
517 small part of the community as described before (5, 24, 51). The surfactant molecules were
518 incorporated in the cell wall and the autolysins, as described for *E. coli* (15), were not or only
519 partly activated. This led to the death of a minority and caused a release of nucleic acids,
520 which stayed bound on the microbes. These nucleic acids may be taken up by the surviving
521 major part of the bacterial community, as described before (48).
522 At high surfactant concentrations a fast autolysis within seconds was obvious, probably due to
523 interaction of the surfactant molecules with the cytoplasma membrane. A possibility would be
524 unspecific interaction of the surfactants in the cell membrane (17), causing a devastation of
525 the cytoplasma membrane (12). A further possibility at low surfactant concentrations is an
526 induced cell lysis of a minor part of the bacterial population probably caused by activating of
527 the autolysin similar molecules in the bacterial cytoplasma membrane. By incorporation of
528 only a few surfactant molecules in the membrane, the autolysin similar molecules may be
529 activated due to a change in the membrane potential. For SDS the change in the membrane
530 potential was described before (2), which results in the activation of autolysins (3) causing
531 pores in the membrane. These pores were indirectly detected in the fluorescence staining by
532 incorporation of the propidium iodide in the cell, allowing the release of proteins and nucleic
533 acids. The nucleic acid uptake would be performed by the major part of the community, as
534 seen in biofilm development (1). At very low surfactant concentrations, measured by an
535 increase of the OD_{600}, the pores in the cytoplasma membrane are probably small that only
536 small signal molecules were released. These molecules may trigger the death or the inhibition
537 of a part of the community but not lysis, as found in growth experiments in stationary phase.

538 For *S. pneumoniae* peptides of the two- component system VncR/S were described (32),
539 causing the activation of a cell death program.
540
541 **Reduced temperature did not always reduce bioleaching velocity**
542 The bioleaching was started in 20 L and 20 mL columns at all tested temperatures. In the
543 small scale columns washed with rainwater a decreasing sulphate release with lowering the
544 temperature was observed. This temperature dependency of the small columns had no effect
545 to the inhibition of the bioleaching in the surfactant treated samples. In the large scale
546 columns operated at 15 to 21°C or at constant 12°C a temperature dependency was only found
547 at the start of the experiments, measured by a lower decrease of the pH value. After the start
548 of the bioleaching the dependency was not observed anymore. This can be explained by the
549 development of a biofilm in the column producing a microhabitat with elevated temperatures
550 probably due to oxidation processes, as described for coal conversion in heap areas in
551 Germany with elevated temperatures above 80°C (53). For low grade copper mines increased
552 sand temperatures, between 60 and 80°C, due to the oxidation of pyrite were described (4).
553 The differences in the behaviour of small and large columns can be explained by a better heat
554 transport from the columns into the environment, resulting in lower temperatures in the small
555 columns. Another factor might be a change in the composition of the population due to the
556 lower sand temperature (9). A further possibility might be the occurrence of original
557 psychrotolerant species stored in the samples, as described for *A. ferrooxidans* strains in
558 columns or stirred tank reactors (7).
559
560 **Inhibition of bioleaching in aquifer material**
561 The application of surfactants to leaching bacteria, living in a biofilm and in the planktonic
562 state, in the columns filled with sand resulted in the inhibition of bioleaching. In the small
563 scale columns the inhibition of the leaching microorganisms was achieved by a single

564 application of the surfactants at the start of the experiments. The inhibition in the leaching
565 solution and the sand lasted for more than 35 weeks, which is similar to results shown before
566 (46). The detection of leaching bacteria in the first run with the large scale columns, despite of
567 treatment with Texapon N70, is explainable by the biodegradation of the surfactant (54).
568 Another possibility might be the adsorption of the surfactant onto the solid particles e.g.
569 lignite, as described for river sediment (44). Therefore the inhibitory effect was not achieved
570 and only temporarily inhibition was found. The incomplete inhibition of leaching
571 microorganisms was also described for 65 m^3 percolators, were only a decrease in cell count
572 was achieved by SDS treatment (41). The following SDS addition to the columns resulted in
573 the complete inhibition of leaching bacteria, revealed that a resistance mechanisms was not
574 involved in this process. The second and third run with the 20 L columns revealed a long time
575 inhibition of the leaching microorganisms at temperatures found in the unaffected overburden.
576 This was achieved only by the treatment with the surfactants SDS or Texapon N70,
577 respectively. For the long term inhibition of the AMD in heap material further treatments with
578 e.g. isothiazolinone or organic material were necessary (40). This indicates the importance of
579 a sufficient high surfactant concentration because of the consumption or strong adsorption to
580 lignite or mineral particles (44).
581
582 For the monitoring of the inhibition of the leaching microorganisms in the sand, due to the
583 surfactant treatment, further experiments have to be performed. For the understanding of the
584 surfactant induced lysis and consequently the way of the successful inhibition of leaching
585 microorganisms, the autolytic enzymes of these bacteria should be identified. Furthermore,
586 the influence of the surfactant treatment to the activity of these enzymes may be examined in
587 following experiments. Due to the lysis of the bacteria the formed fragments may be analyzed
588 and might play a potential role for the monitoring of the inhibition of the microorganisms in
589 the sand material.

Acknowledgement

We are grateful to Peter Jollas (Mitteldeutsche Braunkohlengesellschaft mbH), Ingolf Arnold and Thomas Koch (Vattenfall Europe Mining AG) and Michael Strzodka (GMB GmbH) for the provision of sand samples and for financial support. Furthermore, we would like to thank Gerhard Heide and Reinhard Kleeberg from the TU Bergakademie Freiberg and Harald Euler and Bruno Barbier from the University Bonn for mineralogical analyses of the sand samples. We are grateful to Silvia Boelke for technical assistance.

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793 ## Figure Legends

794

795 **Fig. 1**

796 **Process of sampling of aquifer material in an open lignite mining area in Lusatia. The**
797 **upper 50 cm dry sand layer was removed for the sampling of unaffected material (A).**
798 **For sampling the liner were pressed around one meter in the aquifer material (B).**
799 **Around one ton of material was taken, stored in liners with dry ice for displacing of**
800 **atmospheric oxygen (C and D).**

801

802 **Fig. 2**

803 **Columns in 20 mL (left) and 20 L (right) scale filled with aquifer material, washed with**
804 **rainwater or rainwater supplemented with surfactants. Incubation occurred between 6**
805 **and 12 months between 12 and 21°C.**

806

807 **Fig. 3**

808 **Time course of total cell number determined by MPN- technique from leaching solution**
809 **of six independent 20 L columns. Columns were operated at 15 to 21°C in the first run**
810 **with rainwater (-○-), treated with Texapon N70 and SDS (-●-), in the second run**
811 **washed with rainwater (-□-), treated with Texapon N70 (-■-) and operated in the third**
812 **run at constant 12°C washed with rainwater (-△-), treated with Texapon N70 (-▲-). The**
813 **arrows show the time of surfactant treatment. The cultivation for MPN- experiments**
814 **was performed at 28°C in medium for iron- or sulphur- oxidizing microorganisms for**
815 **three to four weeks, as described before (16, 26).**

816

817 **Fig. 4**

Time course of Fe(II) related to total iron measured from leaching solution of 20 L columns. The columns of the second run were operated at temperatures of 15 to 25°C and washed with rainwater (□) or treated with Texapon N70 (■). The experiment of the third run was performed at constant 12°C and columns were washed either with rainwater (△) or treated with Texapon N70 (▲).

Fig. 5

Time course of total sulphate release from 20 L columns operated at temperatures of 15 to 21°C and washed with rainwater (□) or treated with Texapon N70 (■). The experiment of the third run was performed at constant 12°C and columns were washed either with rainwater (△) or treated with Texapon N70 (▲).

Fig. 6A

Total cell number for iron- plus sulphur- oxidizing microorganisms determined by most-probable- number technique from leaching solutions of 12 independent 20 mL columns. Experiments performed at temperatures of 21°C (top), 15 to 21°C (middle) and constant 12°C (bottom). Columns were washed either with rainwater (□), or treated once with SDS (▨), Texapon N70 (▤), or a mixture of SDS and Texapon N70(▥) . Cultivation for the MPN experiments was performed at 28°C in medium for iron- or sulphur- oxidizing microorganisms for three to four weeks, as described before (16, 26).

Fig. 6B

Sulphate concentrations detected in the wash solution at different time points of 12 parallel 20 mL columns operated at 21°C, 15 to 21°C, or at constant 12°C. The columns were washed either with rainwater (□), or once with SDS (▨), Texapon N70 (▤), or a mixture of SDS and Texapon N70 (▥) and afterwards with rainwater.

844

845 **Fig. 7**

846 **Growth inhibition of *Acidiphilium cryptum* DSM 2389 cultured for one week after**
847 **addition of 10 g/L SDS (left test tube). The right test tube shows a control without SDS**
848 **addition.**

849

850 **Fig. 8**

851 **Time course of OD_{600} after injection of SDS to concentrated suspensions of five different**
852 **bacteria. After 100 seconds of equilibration, SDS was added to a final concentration of**
853 **10 g/L (arrow depicts time of SDS addition and mixing the suspensions in the**
854 **photometer). The cell density was measured for 900 seconds and recorded over time for**
855 ***E. coli* (◇), *B. subtilis* (◆), *Ac. cryptum* DSM 2389 (△), *A. thiooxidans* DSM 622 (○) and**
856 ***A. ferrooxidans* ATCC 23270 (□). Negative controls (−×−) showed a stable OD_{600} for all**
857 **species. The shown data are typical examples of independent experiments.**

858

859 **Fig. 9**

860 **Agarose gels of nucleic acids released into the supernatant or pellet fraction of *E. coli***
861 **(A) or *Ac. cryptum* (B) after SDS (left) or Texapon N70 (right) treatment. After**
862 **photometer assay, the supernatant (S) and pellet (P) fraction was separated on a 0.8%**
863 **agarose gel. Cells incubated in water were used as negative controls. Lanes: (left) high**
864 **range marker (Fermentas); (1) negative control; (2) 1 mg/L SDS; (3) 5 mg/L SDS; (4) 25**
865 **mg/L SDS; (5) 50 mg/L SDS; (6) 250 mg/L SDS; (7) 500 mg/L SDS; (8) 1 g/L SDS; (9) 2**
866 **g/L SDS; (10) 5 g/L SDS; (11) 10 g/L SDS; (right) middle range marker (Fermentas).**

867

868

869 **Fig. 10**

870 **Characterisation of nucleic acids released from *E. coli* (A) or *Ac. cryptum* (B) by DNase**
871 **or RNase digestion. After SDS treatment the pellet fraction was separated on a 0.8%**
872 **agarose gel. Cells incubated in water were used as negative controls (c). Cells incubated**
873 **with 10 g/L SDS or Texapon N70 were used as positive controls. The cell suspension was**
874 **incubated separately with DNase or RNase after treatment with surfactant. For DNase**
875 **control an *Eco*RI digested plasmid Yep352-p25 was used by incubating the plasmid with**
876 **DNase.**

877

878 **Fig. 11**

879 **Release of protein after treatment with SDS. The protein released from *E. coli* by**
880 **grinding with liquid N_2 was set to 100%. The released protein per cell for *E. coli* was**
881 **used for determination of the released amount of protein of the other bacteria calculated**
882 **to the size of the cells. *E. coli* (—■—), *Ac. cryptum* (▭), *A. thiooxidans* (▤) and *A.**
883 ***ferrooxidans* (▨).**

884

885 **Fig.12**

886 **Fluorescence micrograph of *E. coli* (I) and *Ac. cryptum* (II) cultures indicating**
887 **membrane status. Living control cells (A), isopropanol-killed cells (B) and SDS treated**
888 **cells (C) were stained with a mixture of 6 µM SYTO9 and 30 µM propidium iodide**
889 **fluorescent viability dyes. SYTO9 is a green fluorescent membrane permeant dye**
890 **staining all cells, in case they contain nucleic acid. Propidium iodide is a red fluorescent**
891 **intercalating dye which is membrane impermeant and consequently enters only cells**
892 **with damaged cytoplasmic membranes. Both stains were analysed separately and the**
893 **micrographs were merged. *E. coli* cells treated with SDS showed green or red**

894 **fluorescence, whereas cells of *Ac. cryptum* showed the same behaviour as isopropanol**
895 **treated cells. Bar = 10 µm**
896
897 **Fig. 13**
898 **Fluorescence spectrometric analysis of SDS treated *E. coli* (A), *Ac. cryptum* (B), *A.***
899 ***ferrooxidans* (C), *A. thiooxidans* (D) indicating permeability of the plasma membrane.**
900 **Bacterial cells were treated with water (alive;), 70% isopropanol (dead;) and**
901 **concentrations of 10 g/L (), or 0.25 g/L () SDS. Each cell sample was stained**
902 **with a mixture of 6 µM SYTO9 plus 30 µM PI and fluorescence emission spectrum was**
903 **analyzed on a fluorescence spectrophotometer. The spectra were measured with**
904 **excitation at 470 nm.**

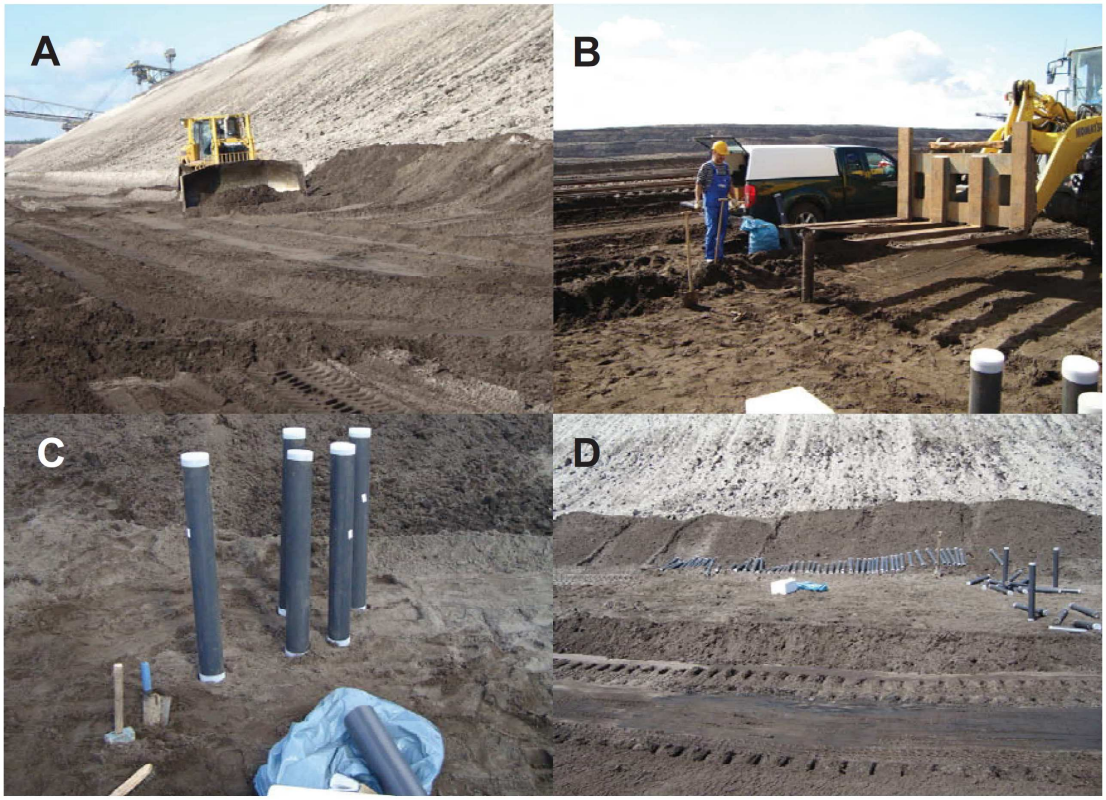

Figure 1

Process of sampling of aquifer material in an open lignite mining area in Lusatia. The upper 50 cm dry sand layer was removed for the sampling of unaffected material (A). For sampling the liner were pressed around one meter in the aquifer material (B). Around one ton of material was taken, stored in liners with dry ice for displacing of atmospheric oxygen (C and D).

Figure 2

Columns in 20 mL (left) and 20 L (right) scale filled with aquifer material, washed with rainwater or rainwater supplemented with surfactants. Incubation occurred between 6 and 12 months between 12 and 21°C.

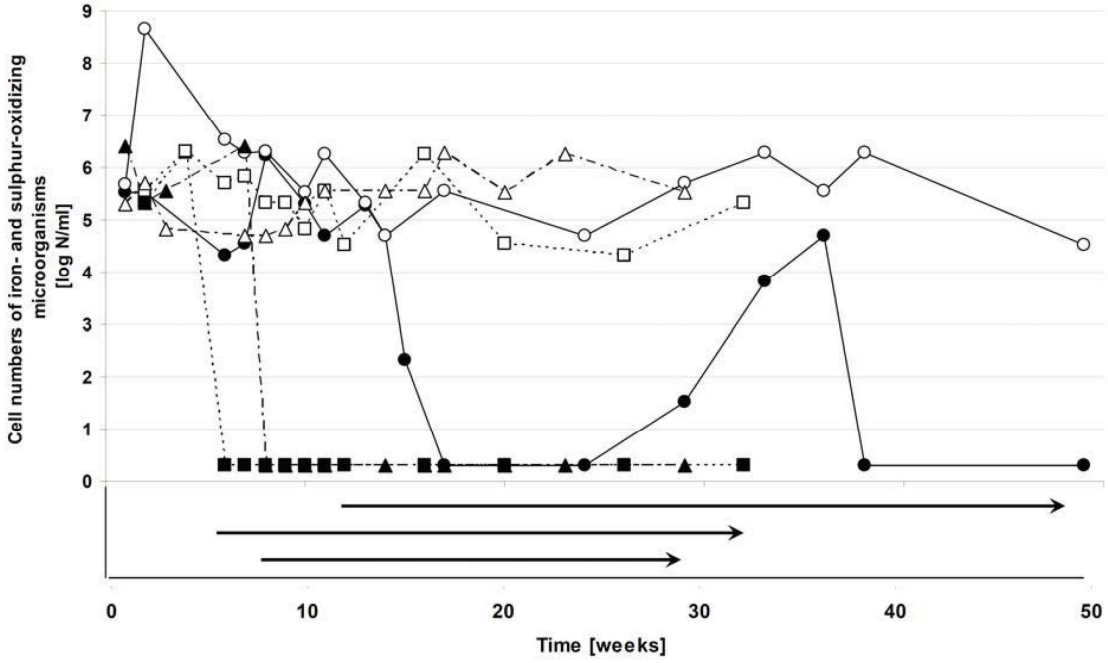

Figure 3

Time course of total cell number determined by MPN- technique from leaching solution of six independent 20 L columns. Columns were operated at 15 to 21°C in the first run with rainwater (-○-), treated with Texapon N70 and SDS (-●-), in the second run washed with rainwater (-□-), treated with Texapon N70 (-■-) and operated in the third run at constant 12°C washed with rainwater (-△-), treated with Texapon N70 (-▲-). The arrows show the time of surfactant treatment. The cultivation for MPN- experiments was performed at 28°C in medium for iron- or sulphur- oxidizing microorganisms for three to four weeks, as described before (16, 26).

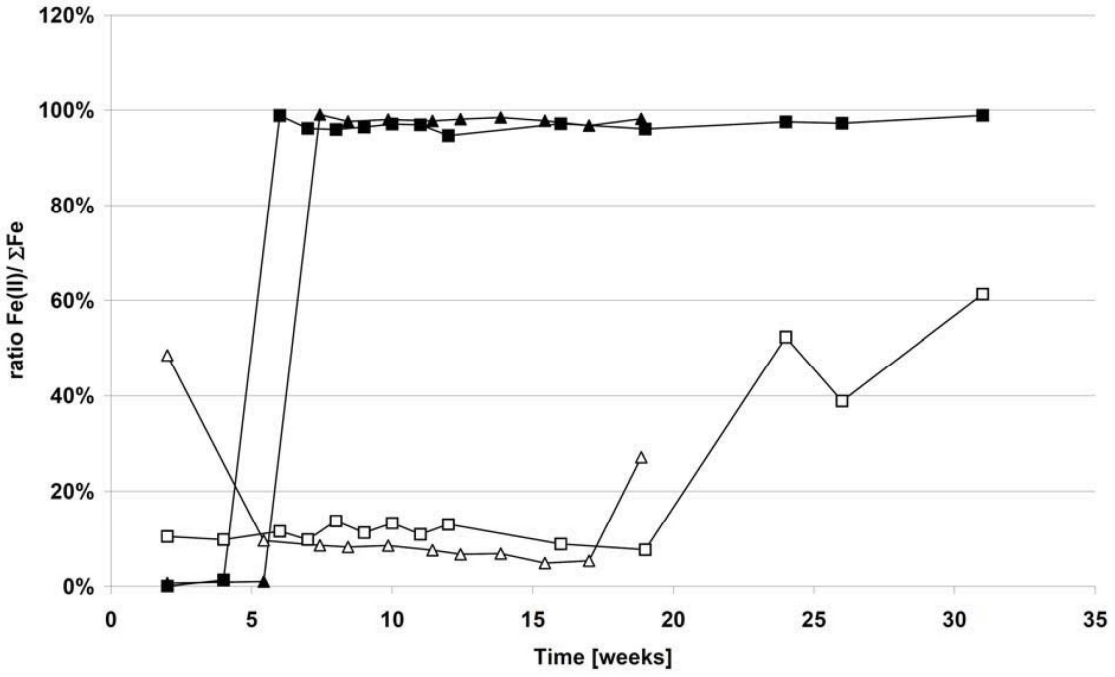

Figure 4

Time course of Fe(II) related to total iron measured from leaching solution of 20 L columns. The columns of the second run were operated at temperatures of 15 to 25°C and washed with rainwater (-□-) or treated with Texapon N70 (-■-). The experiment of the third run was performed at constant 12°C and columns were washed either with rainwater (-△-) or treated with Texapon N70 (-▲-).

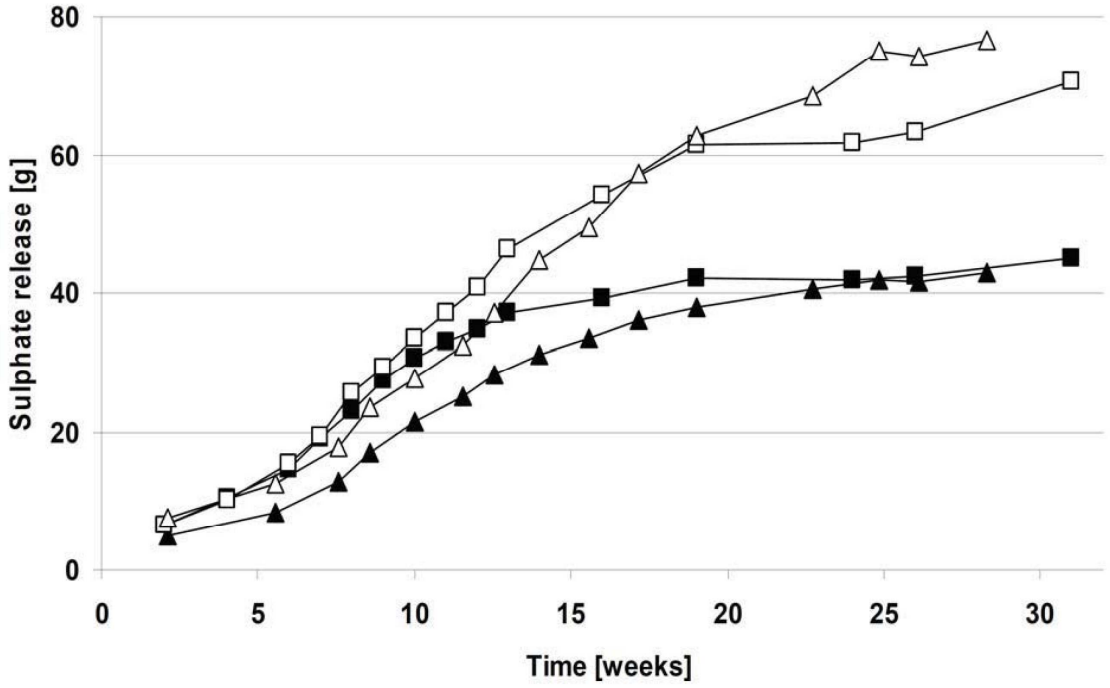

Figure 5

Time course of total sulphate release from 20 L columns operated at temperatures of 15 to 21°C and washed with rainwater (-□-) or treated with Texapon N70 (-■-). The experiment of the third run was performed at constant 12°C and columns were washed either with rainwater (-△-) or treated with Texapon N70 (-▲-).

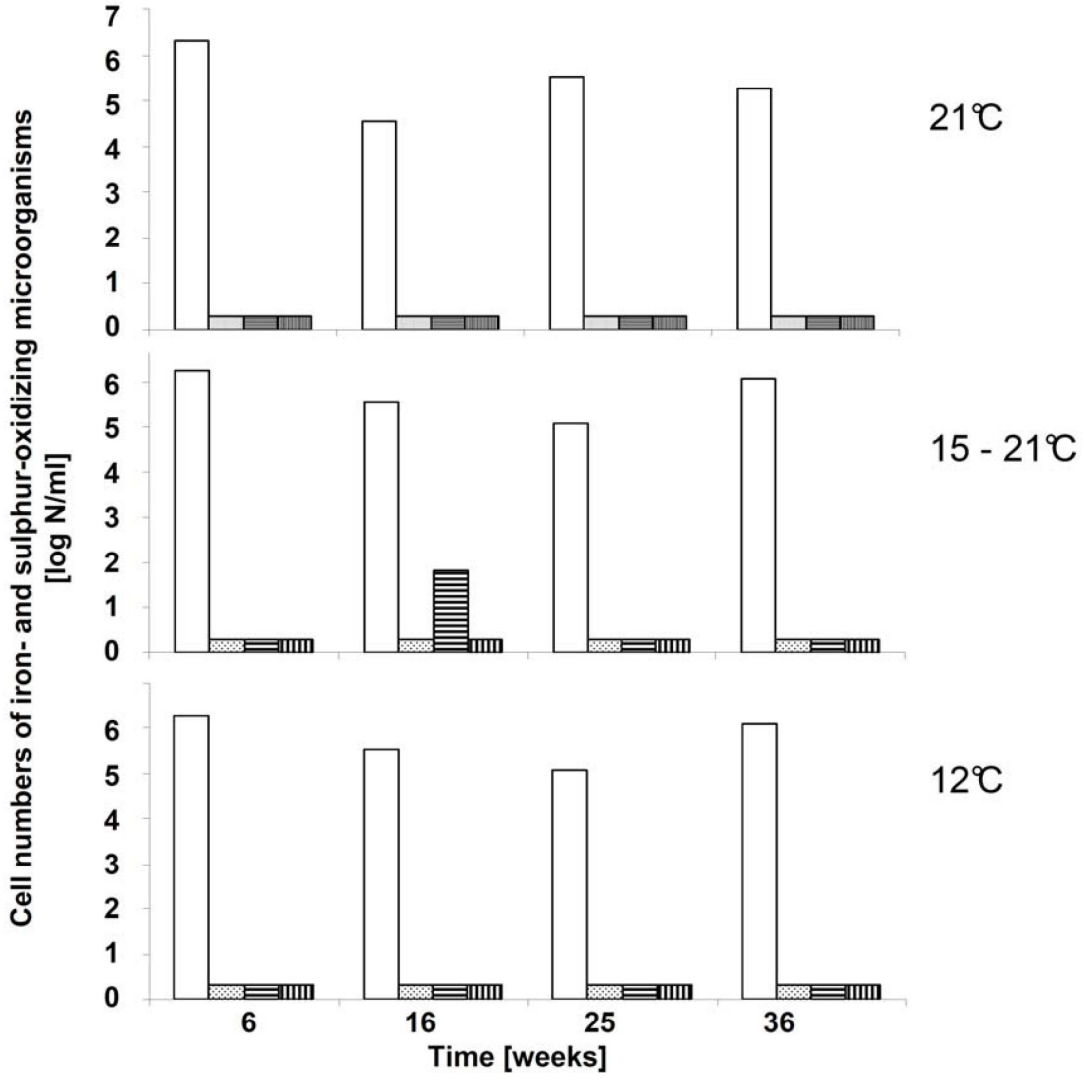

Figure 6A

Total cell number for iron- plus sulphur- oxidizing microorganisms determined by most-probable- number technique from leaching solutions of 12 independent 20 mL columns. Experiments performed at temperatures of 21°C (top), 15 to 21°C (middle) and constant 12°C (bottom). Columns were washed either with rainwater (□), or treated once with SDS (▨), Texapon N70 (▤), or a mixture of SDS and Texapon N70(▥) . Cultivation for the MPN experiments was performed at 28°C in medium for iron- or sulphur- oxidizing microorganisms for three to four weeks, as described before (16, 26).

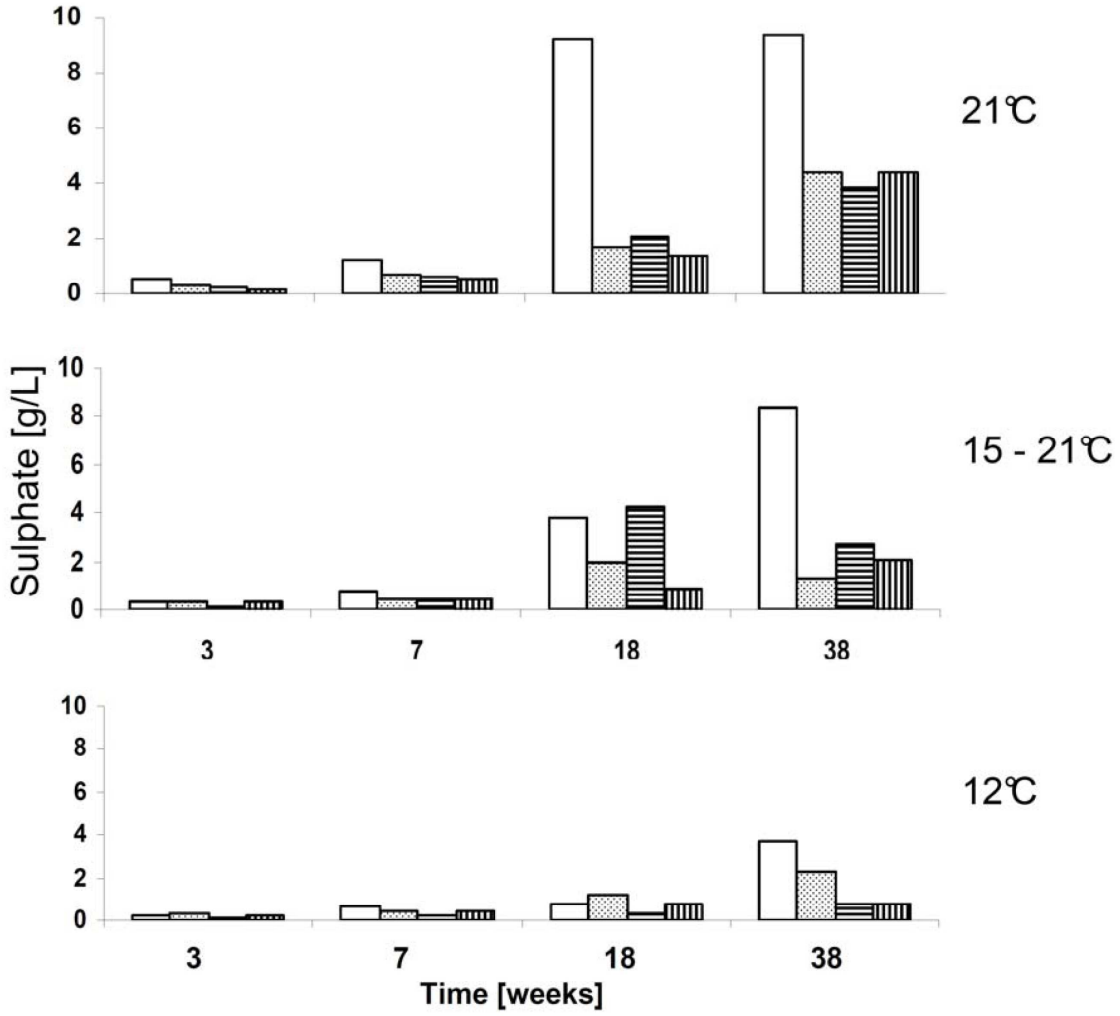

Figure 6B

Sulphate concentrations detected in the wash solution at different time points of 12 parallel 20 mL columns operated at 21°C, 15 to 21°C, or at constant 12°C. The columns were washed either with rainwater (□), or once with SDS (▨), Texapon N70 (▤), or a mixture of SDS and Texapon N70 (▥) and afterwards with rainwater.

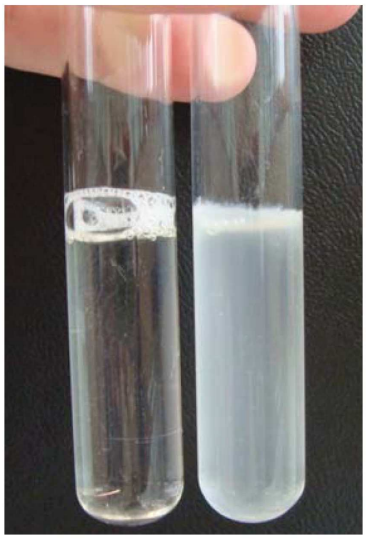

Figure 7

Growth inhibition of *Acidiphilium cryptum* DSM 2389 cultured for one week after addition of 10 g/L SDS (left test tube). The right test tube shows a control without SDS addition.

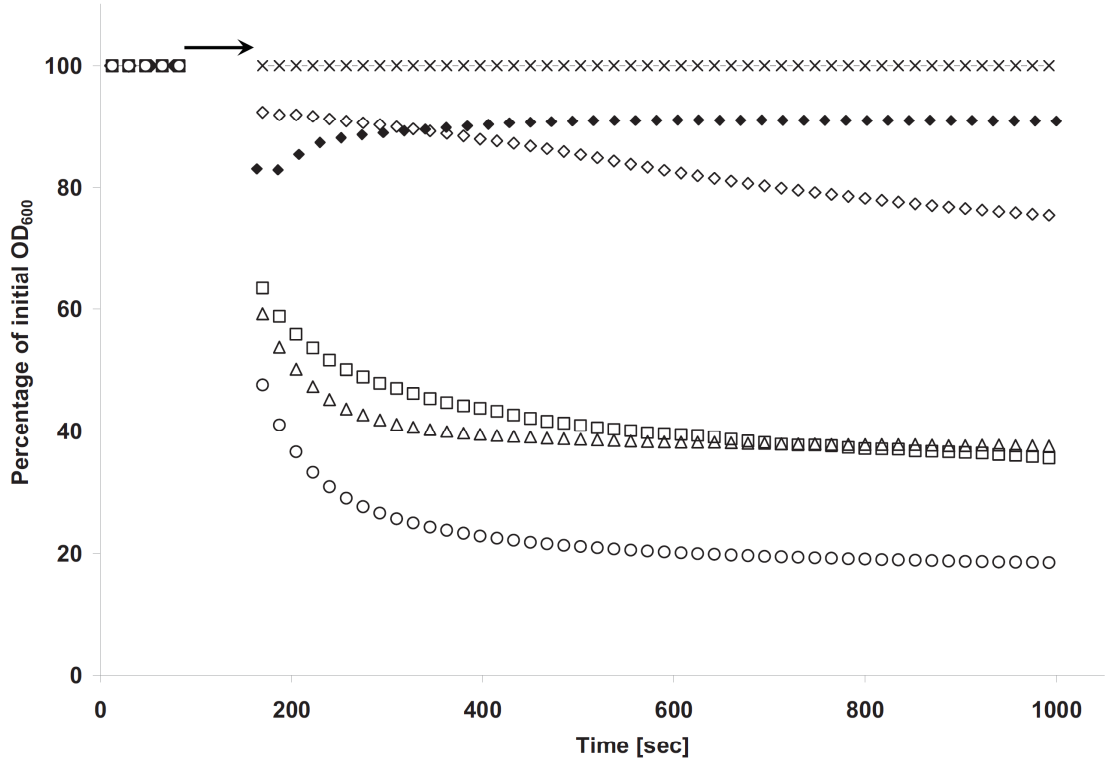

Figure 8

Time course of OD_{600} after injection of SDS to concentrated suspensions of five different bacteria. After 100 seconds of equilibration, SDS was added to a final concentration of 10 g/L (arrow depicts time of SDS addition and mixing the suspensions in the photometer). The cell density was measured for 900 seconds and recorded over time for *E. coli* (◇), *B. subtilis* (◆), *Ac. cryptum* DSM 2389 (△), *A. thiooxidans* DSM 622 (○) and *A. ferrooxidans* ATCC 23270 (□). Negative controls (—×—) showed a stable OD_{600} for all species. The shown data are typical examples of independent experiments.

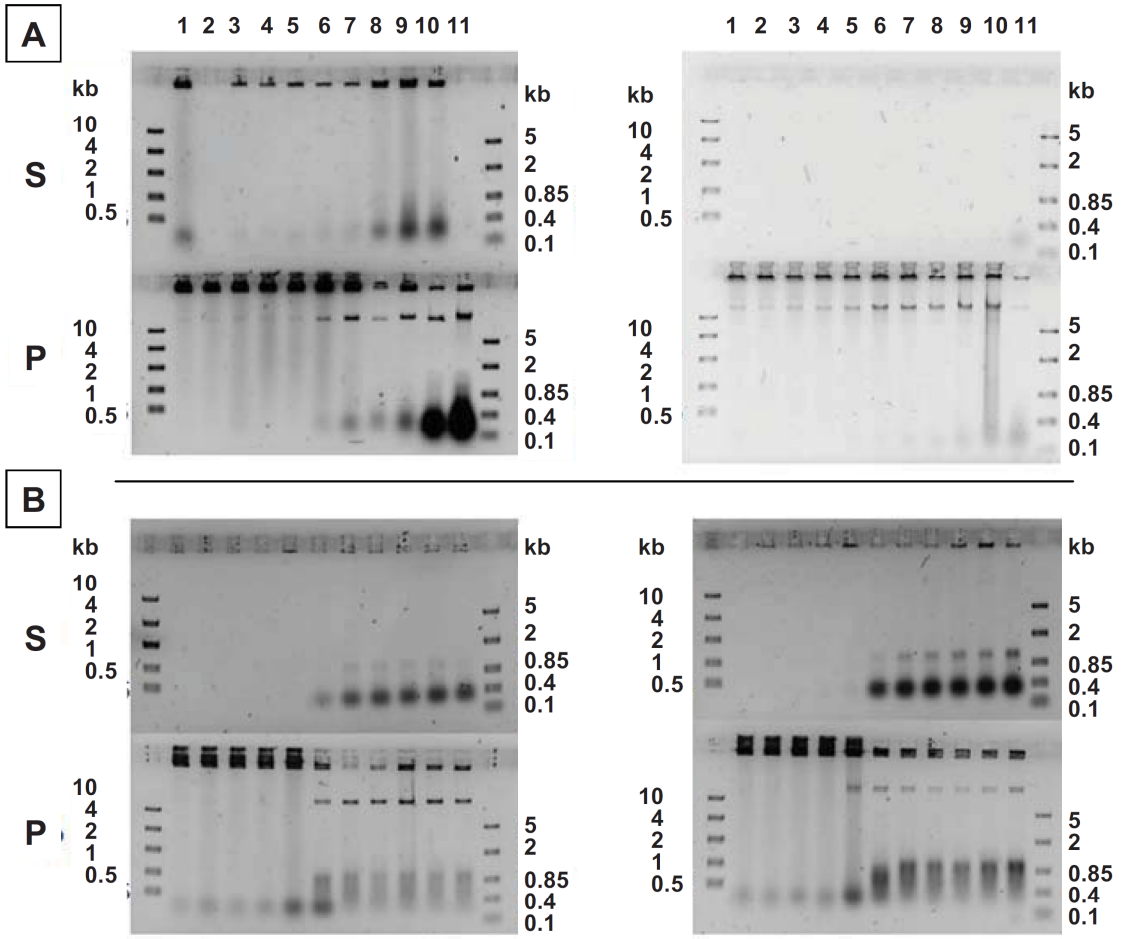

Figure 9

Agarose gels of nucleic acids released into the supernatant or pellet fraction of *E. coli* (A) or *Ac. cryptum* (B) after SDS (left) or Texapon N70 (right) treatment. After photometer assay, the supernatant (S) and pellet (P) fraction was separated on a 0.8% agarose gel. Cells incubated in water were used as negative controls. Lanes: (left) high range marker (Fermentas); (1) negative control; (2) 1 mg/L SDS; (3) 5 mg/L SDS; (4) 25 mg/L SDS; (5) 50 mg/L SDS; (6) 250 mg/L SDS; (7) 500 mg/L SDS; (8) 1 g/L SDS; (9) 2 g/L SDS; (10) 5 g/L SDS; (11) 10 g/L SDS; (right) middle range marker (Fermentas).

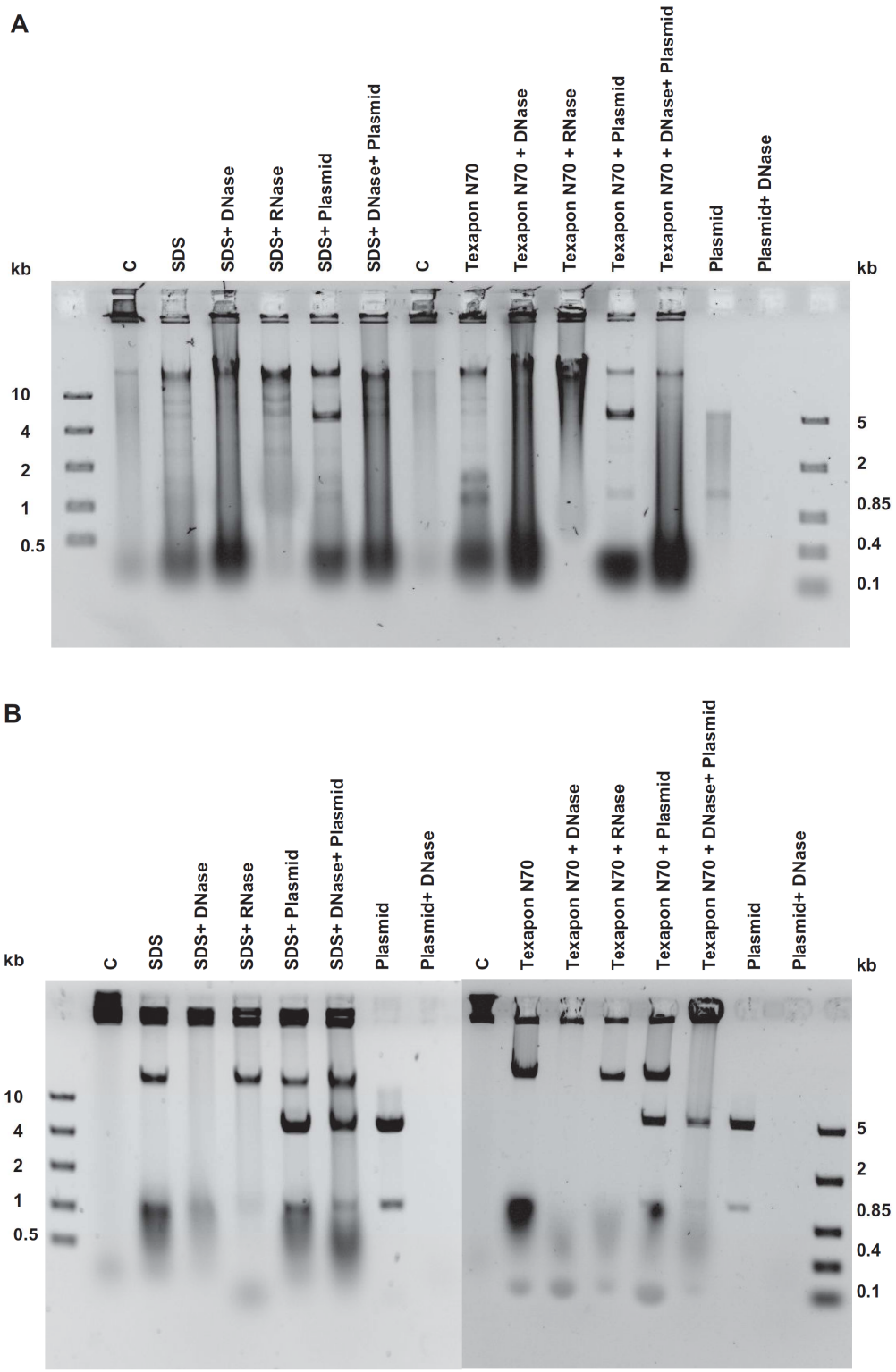

Figure 10

Characterisation of nucleic acids released from *E. coli* (A) or *Ac. cryptum* (B) by DNase or RNase digestion. After SDS treatment the pellet fraction was separated on a 0.8%

agarose gel. Cells incubated in water were used as negative controls (c). Cells incubated with 10 g/L SDS or Texapon N70 were used as positive controls. The cell suspension was incubated separately with DNase or RNase after treatment with surfactant. For DNase control an *Eco*RI digested plasmid Yep352-p25 was used by incubating the plasmid with DNase.

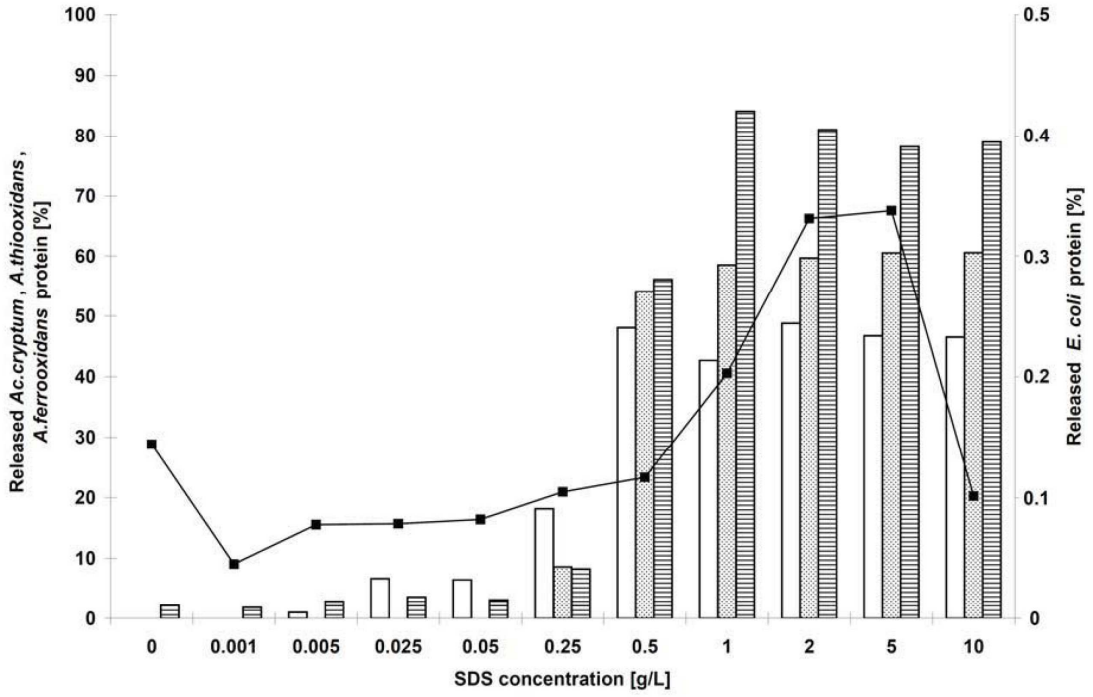

Figure 11

Release of protein after treatment with SDS. The protein released from *E. coli* by grinding with liquid N_2 was set to 100%. The released protein per cell for *E. coli* was used for determination of the released amount of protein of the other bacteria calculated to the size of the cells. *E. coli* (–■–), *Ac. cryptum* (▭), *A. thiooxidans* (▤) and *A. ferrooxidans* (▨).

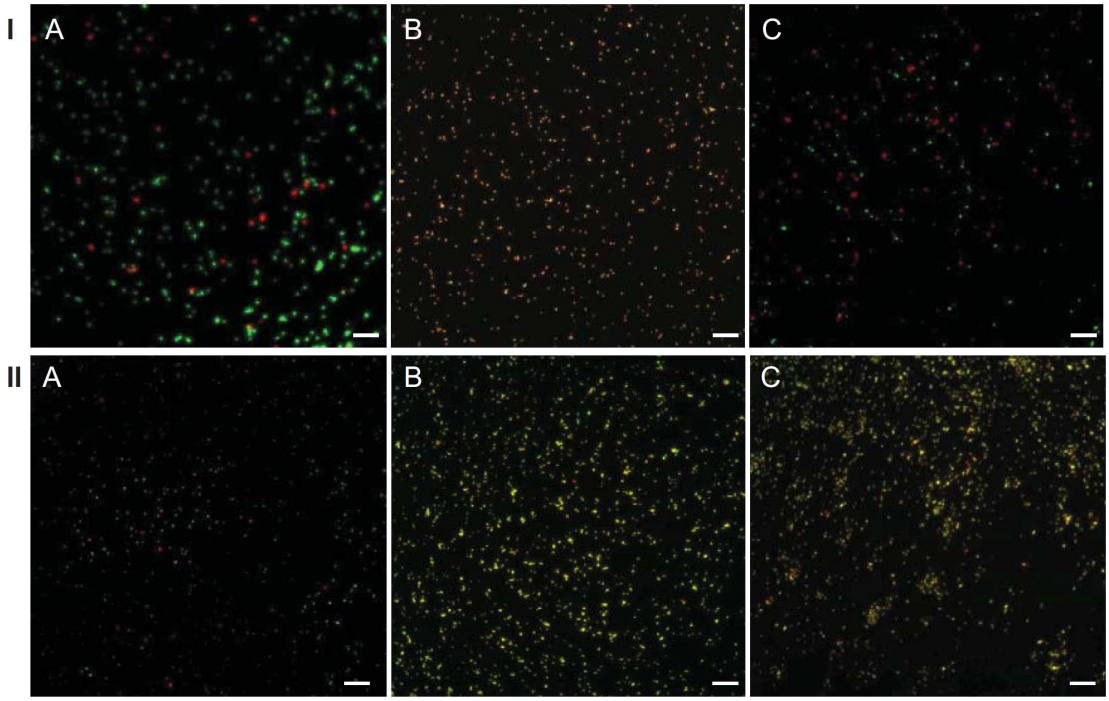

Figure 12

Fluorescence micrograph of *E. coli* (I) and *Ac. cryptum* (II) cultures indicating membrane status. Living control cells (A), isopropanol-killed cells (B) and SDS treated cells (C) were stained with a mixture of 6 µM SYTO9 and 30 µM propidium iodide fluorescent viability dyes. SYTO9 is a green fluorescent membrane permeant dye staining all cells, in case they contain nucleic acid. Propidium iodide is a red fluorescent intercalating dye which is membrane impermeant and consequently enters only cells with damaged cytoplasmic membranes. Both stains were analysed separately and the micrographs were merged. *E. coli* cells treated with SDS showed green or red fluorescence, whereas cells of *Ac. cryptum* showed the same behaviour as isopropanol treated cells. Bar = 10 µm

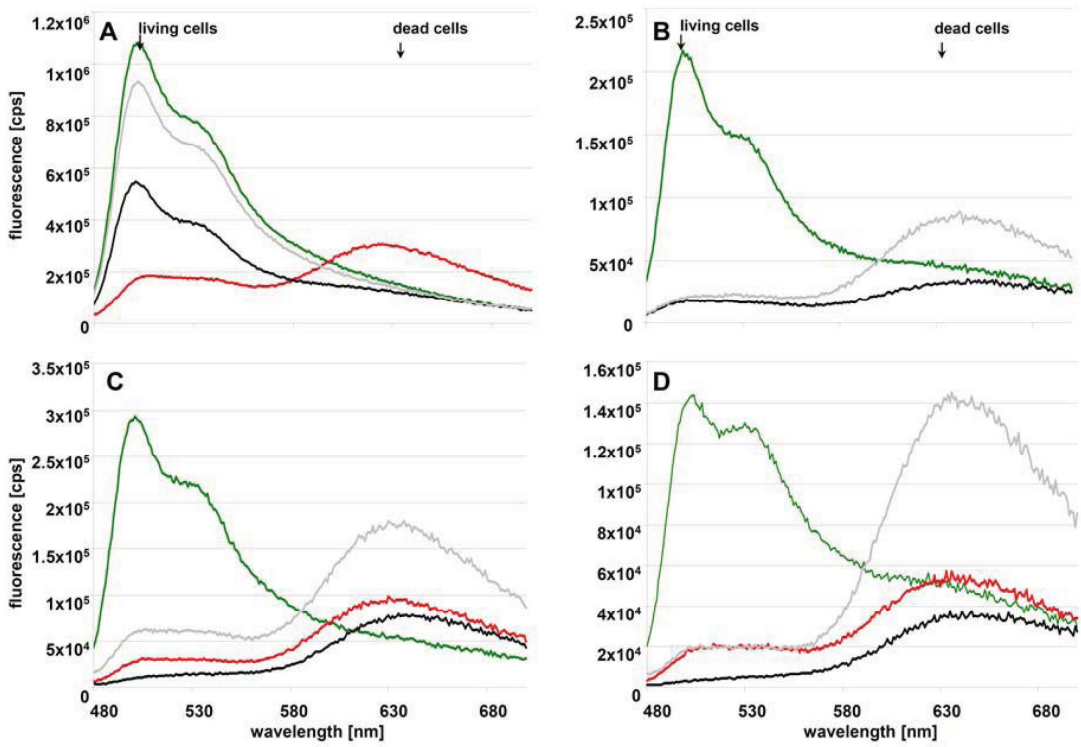

Figure 13

Fluorescence spectrometric analysis of SDS treated *E. coli* (A), *Ac. cryptum* (B), *A. ferrooxidans* (C), *A. thiooxidans* (D) indicating permeability of the plasma membrane. Bacterial cells were treated with water (alive;), 70% isopropanol (dead;) and concentrations of 10 g/L (), or 0.25 g/L () SDS. Each cell sample was stained with a mixture of 6 µM SYTO9 plus 30 µM PI and fluorescence emission spectrum was analyzed on a fluorescence spectrophotometer. The spectra were measured with excitation at 470 nm.

Tab. 1.

Concentration of SDS or Texapon N70 needed to inhibit growth of planktonic cells of the four specis *E. coli*, *Ac. cryptum* DSM 2389, *A. thiooxidans* DSM 622, *A. ferrooxidans* ATCC 23270.

Surfactant	*E. coli*	*Ac. cryptum*	*A. thiooxidans*	*A. ferrooxidans*
SDS [g/L]	0.5	0.025	0.025	0.005
Texapon N70 [g/L]	0.5	0.05	0.025	0.005

Tab. 2.

Minimal concentration of the two surfactants SDS and Texapon N70 needed for total clearance of cultures in test tubes which were turbid after two days to one week, according to growth rates. Examined were planktonic cells of the four specis *E. coli*, *Ac. cryptum* DSM 2389, *A. thiooxidans* DSM 622, *A. ferrooxidans* ATCC 23270 in stationary phase.

Surfactant	*E. coli*	*Ac. cryptum*	*A. thiooxidans*	*A. ferrooxidans*
SDS [g/L]	1.0	0.5	0.5	0.5
Texapon N70 [g/L]	> 10.0	2.0	1.0	2.0

Poster und Vorträge

Hans-Michael Siebert, Tobias Uhlig, Sylvia Hondke, Wolfgang Sand, and K.-Peter Stahmann. 2011. A Lusatian open cast lignite mine - a source of new iron-oxidizing microorganisms? In: 19th International Biohydrometallurgy Symposium - IBS 2011, Changsha, China, 17.- 25.09.2011. Vortrag

Hans-Michael Siebert, Yvonne Wloka, Wolfgang Sand, and K.-Peter Stahmann. 2011. Fast Response of High Density Planktonic Leaching Microorganisms to Growth Inhibiting Tensids. In: BIOspektrum- Tagungsband zur VAAM Jahrestagung 2011. Karlsruhe. 03.- 06.04.2011. Poster Präsentation

Hans-Michael Siebert, Robert Marmulla, and K.-Peter Stahmann. 2010. Effect of SDS on planctonic *Acidithiobacillus thiooxidans* and bioleaching of sand samples. In: Bio and Hydromet´10, Cape Town, South Africa. 08.- 09.11.2010. Vortrag

Hans-Michael Siebert, Thore Rohwerder, Wolfgang Sand, Michael Strzodka and K.-Peter Stahmann. 2009. Evidence for iron- and sulfur-oxidizing bacteria and archaea in a currently active lignite mining area of Lusatia (eastern Germany). In: 18th International Biohydrometallurgy Symposium - IBS 2009, Bariloche, Argentina, 13.- 17.09.2009. Poster Präsentation

5. Anhang

5.1 Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

5.2 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Bioleaching im Lausitzer und Mitteldeutschen Braunkohlerevier- Mikrobiologische Bestandsaufnahme und Laborexperimente zur Hemmung der mikrobiellen Pyrit-Oxidation”

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im August 2012

5.3 Danksagung

Die vorliegende Arbeit wurde an der Fakultät für Naturwissenschaften der Hochschule Lausitz in Zusammenarbeit mit dem Biofilmcentre der Universität Duisburg- Essen durchgeführt und durch Mitteldeutsche Braunkohlen-gesellschaft mbH, Vattenfall Europe Mining AG und GMB GmbH finanziert.

Herrn Prof. Stahmann danke ich für die Überlassung dieses interessanten Themas und seine fortwährende und bereitwillige Unterstützung. Weiterhin möchte ich mich für die vielen Diskussionen und Anregungen bedanken.

Herrn Prof. Sand möchte ich für die Übernahme des Referates, seine dauerhafte Unterstützung und die Bereitschaft zur konstruktiven Kritik danken.

Ich möchte mich weiterhin bei Herrn Dr. Peter Jollas von der Mitteldeutschen Braunkohlengesellschaft mbH, bei Herrn Ingolf Arnold und Herrn Dr. Thomas Koch von der Vattenfall Europe Mining AG und Herrn Dr. Michael Strzodka von der GMB GmbH für die Finanzierung dieses Projektes und die Unterstützung bei den Probennahmen bedanken.

Herrn Dr. Schörken von der Cognis GmbH danke ich für die wissenschaftliche Betreuung bezüglich der ausgewählten Tenside.

Ein weiterer Dank geht an Herrn Prof. Heide und Herrn Dr. Kleeberg von der TU Bergakademie Freiberg, sowie Herrn Dr. Euler und Herrn Barbier von der Universität Bonn für die Durchführung der Pyrit- Analysen.

Herrn Prof. Acker und Herrn Prof. Pulz möchte ich für die Durchführung der Elementar- Analysen der Tagebauproben danken.

Mein Dank geht auch an die Mitarbeiter des Arbeitskreises von Prof. Sand, insbesondere an Bianca Florian für die Unterstützung und Diskussion während der Arbeit.

Im Besonderen möchte ich mich bei Susann Barig, Susanne Nieland, Glenn Gröbe und Tino Koncz für das nette Arbeitsklima bedanken.

Bei den Professoren und Mitarbeitern der Fakultät für Naturwissenschaften der Hochschule Lausitz möchte ich mich für die freundliche Hilfsbereitschaft und gute Zusammenarbeit bedanken.

Ein Dank geht auch an meine studentischen Hilfskräfte Yvonne, Julia, Katja, Linda, Silvia, Hannes, Katrin, Robert, Lisa Maria und Susann, die mich in der Zeit meiner Promotion tatkräftig unterstützt haben.